

November 2015

In Vitro Screening for Small-Molecule Kinase Inhibitors of Testis-Specific Serine Kinase 1 (TSSK1), a Potential Target for Male Contraception

Julie Majka
University of Massachusetts Amherst

Follow this and additional works at: https://scholarworks.umass.edu/masters_theses_2

Recommended Citation

Majka, Julie, "In Vitro Screening for Small-Molecule Kinase Inhibitors of Testis-Specific Serine Kinase 1 (TSSK1), a Potential Target for Male Contraception" (2015). *Masters Theses*. 284.
<https://doi.org/10.7275/7205169> https://scholarworks.umass.edu/masters_theses_2/284

This Campus-Only Access for Five (5) Years is brought to you for free and open access by the Dissertations and Theses at ScholarWorks@UMass Amherst. It has been accepted for inclusion in Masters Theses by an authorized administrator of ScholarWorks@UMass Amherst. For more information, please contact scholarworks@library.umass.edu.

***IN VITRO* SCREENING FOR SMALL-MOLECULE KINASE INHIBITORS OF TESTIS-
SPECIFIC SERINE KINASE 1 (TSSK1), A POTENTIAL TARGET FOR MALE
CONTRACEPTION**

A Thesis Presented

by

JULIE KATHERINE MAJKA

Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
of the requirements for the degree of

MASTER OF SCIENCE

September 2015

Animal Biotechnology and Biomedical Sciences,
Veterinary and Animal Science Department

***“IN VITRO* SCREENING FOR SMALL-MOLECULE KINASE INHIBITORS OF TESTIS-
SPECIFIC SERINE KINASE 1 (TSSK1), A POTENTIAL TARGET FOR MALE
CONTRACEPTION”**

A Thesis Presented

by

JULIE KATHERINE MAJKA

Approved as to style and content by:

Ana Maria Salicioni, Chair

Rafael Fissore, Member

Katherine Beltaire, Member

Rafael Fissore, Department Head
Veterinary and Animal Sciences Department

DEDICATION

To Drs. Bill Beal and James Knight – two professors who told me to never do anything in the field of reproductive biology because I “would never be any good at it.”

To Dr. Cynthia Wood – standing in front of my Ansci seminar class you took a poll of what we wanted to be when we grew up. You said “54 of you want to be veterinarians; maybe two or three of you will be. Everyone else, find a new dream.” I’m glad I ignored your advice; this thesis is a testament to pursuing your dream even if you’re told that you’ll never achieve it.

To my family and my Matthew – a continuous supply of support, encouragement, and love, –sometimes tough love! –trips to Cindy’s, homemade meals, chauffeuring post-knee surgery, and everything in between made this possible. Thank you for all you’ve done for the past two years.

ACKNOWLEDGEMENTS

I would like to both acknowledge and thank Dr. Ana Maria Salicioni and Dr. Pablo Visconti for allowing me this opportunity to learn and grow as a scientist. Thanks to your dedication and patience, I have gained a great deal of knowledge and have been able to achieve my goal of going to veterinary school.

I would like to thank my committee: Dr. Ana Maria Salicioni, Dr. Rafael Fissore, and Dr. Katherine Beltaire for their willingness to be members of my committee and make time in their schedules for me to complete this thesis.

A special thanks goes to Dr. Maria Gracia Gervasi, without whom I probably would have given up a long time ago. She has the patience of a saint and has been a wonderful teacher, mentor, and friend to me for the past two years. Maria's insistence of thoroughness taught me how to be critical of my work –an invaluable lesson in science, and in life. Her positivity and constant encouragement were sometimes the only things pushing me through this program.

My fellow lab members: Antonio, Felipe, Bidur, and our undergraduate students – thank you all for the help you provided to train me, allowing me to gain experience and ask questions, opening the liquid nitrogen tank, and the camaraderie you've given.

The graduate students of the Veterinary and Animal Sciences department for providing support throughout this degree, for lending an ear when I needed to talk, or work through an experiment or assignment, and for providing guidance when needed. Thank you especially to Margarita, who was my sounding board and a huge support while here at school and while at work. Working six days a week would have been miserable without you!

All of the members of the VASCI department who we would be lost without:
Mary Schneider for making sure I always had my paperwork in order and who can
answer nearly any question you could ever have, Dr. Lisa Minter and Dr. Cynthia
Baldwin for acting as our graduate program directors, members of the business office
who were always there with a smile and an answer, and to all the professors who are so
dedicated to helping students achieve their goals.

ABSTRACT

IN VITRO SCREENING FOR SMALL-MOLECULE KINASE INHIBITORS OF TESTIS-SPECIFIC SERINE KINASE 1 (TSSK1), A POTENTIAL TARGET FOR MALE CONTRACEPTION

SEPTEMBER 2015

**JULIE K. MAJKA, B.S. VIRGINIA POLYTECHNIC INSTITUTE AND STATE UNIVERSITY
M.S., UNIVERSITY OF MASSACHUSETTS, AMHERST**

Directed by: Dr. Ana Maria Salicioni

Despite the availability of a range of contraceptive methods, over 50% of pregnancies are unintended worldwide (~1,000,000/day). Male contraception remains an important area of research. Recent acceptability studies indicate that a high percentage of men would be willing to use a male contraceptive pill. Several non-hormonal targets for a male-based contraceptive pill have been brought to light recently including the testis-specific serine kinase (TSSK) family, which belongs to the AMPK kinase branch in the CAMK group. The finding that TSSK family members are postmeiotically expressed in male germ cells, together with the infertile phenotype found in both *Tssk1/2* and *Tssk6* mouse knock-out models, has led to the proposal that TSSKs play an important role in sperm function, making them excellent targets for drug design of novel male contraceptives.

This research is aimed to the identification of potent, specific inhibitors for TSSK1 activity for the development of novel male contraceptive pills through the use of mice as an experimental model. Our hypothesis is that a single kinase inhibitor can specifically inhibit TSSK's kinase activity without interfering with other sperm functions, or other tissue types. In this study, we have assessed the enzyme properties of

recombinant human TSSK1 by application of a homogeneous, non-radioactive enzyme activity assay and mid-throughput screening of kinase inhibitors that block TSSK1 activity. Some of these inhibitors have proven to greatly reduce the activity of TSSK1 overall, but lack the specificity desired to be plausible for drug design.

In parallel, our efforts are directed towards developing a functional *in vivo* assay using both CD1 and *acrosin-GFP*-transgenic mice (expressing Acr-GFP in the acrosome) based on our finding that TSSK1 is localized in the sperm acrosome, which points to a potential role of TSSKs in sperm acrosome reaction.

The anticipated outcome of this research is to ultimately develop a reversible, non-hormonal male contraceptive. Our findings may also set the basis for studies on human sperm function towards a better understanding of male reproductive physiology and mechanisms of male infertility.

TABLE OF CONTENTS

	Page
ACKNOWLEDGEMENTS.....	iv
ABSTRACT	vi
LIST OF FIGURES	xi
LIST OF ABBREVIATIONS.....	xii
 CHAPTER	
1. INTRODUCTION AND MATERIALS & METHODS	1
1.1 Introduction	1
1.2 Materials & Methods.....	4
1.2.1 Aim 1 Materials	4
1.2.1.1 ADP-Glo™ kit.....	4
1.2.1.2 Kinases and Substrates	5
1.2.1.3 Inhibitors	5
1.2.1.4 Equipment and Data Analysis	5
1.2.2 Aim 1 Methods	6
1.2.2.1 Kinase Assay	6
1.2.3 Aim 2 Materials	7
1.2.3.1 Animals	7
1.2.3.2 Media and Inhibitors	7
1.2.3.3 Western Blot and Immunoprecipitation Assay	8
1.2.3.4 Sperm Cell Viability Assay.....	9
1.2.3.5 Computer-Assisted Semen Analysis (CASA).....	9
1.2.4 Aim 2 Methods	10
1.2.4.1 Protein Extraction.....	10
1.2.4.2 Sperm Capacitation	10
1.2.4.3 Compound C.....	11
1.2.4.4 Western Blot.....	11
1.2.4.4.1 Anti-PKA substrates.....	11

1.2.4.4.2 Anti-PY	12
1.2.4.4.3 Tubulin	12
1.2.4.4.4 AMPK α	12
1.2.4.5 Immunoprecipitation	13
1.2.4.6 CASA	14
1.2.4.7 Acrosome Reaction	14
1.2.4.8 PNA Fluorescence Imaging.....	15
1.2.4.9 Viability	16
1.2.4.10 <i>In Vitro</i> Fertilization (IVF).....	17
1.2.4.11 Statistical Analysis	18
2. <i>IN VITRO</i> SCREENING FOR SMALL-MOLECULE KINASE INHIBITORS OF TESTIS-SPECIFIC SERINE KINASE 1 (TSSK1)	27
2.1 Rationale	27
2.2 Results.....	28
2.2.1 Testing Assay Sensitivity.....	28
2.2.2 Testing Substrate Sensitivity.....	29
2.2.3 rTSSK1 Concentration Curve	29
2.2.4 Testing rTSSK1 Inhibition <i>In Vitro</i>	30
3. <i>IN VITRO</i> TESTING OF SMALL-MOLECULE KINASE INHIBITORS OF AMPK/TSSK	37
3.1 Rationale	37
3.2 Results – Aim 2.1	39
3.2.1 Investigation of AMPK in Murine Sperm	39
3.3 Results – Aim 2.2	41
3.3.1 Effect of Compound C on Sperm Signaling Events	41
3.3.2 Computer-Assisted Sperm Analysis	42
3.3.3 Compound C and the Acrosome Reaction.....	44
3.3.4 <i>In Vitro</i> Fertilization	45
4. CONCLUSIONS, DISCUSSION, & FUTURE DIRECTIONS	53
4.1 Conclusions & Discussion	53
4.2 Future Directions	58

BIBLIOGRAPHY	61
--------------------	----

LIST OF FIGURES

Figure	Page
1: Unintended Pregnancy and Contraceptive Options	19
2: CAMK Branch of the Human Kinome	20
3: Biochemical Events During Sperm Capacitation	21
4: Immunolocalization of TSSK1	22
5: ADP-Glo™ Mechanism	23
6: The Acrosome Reaction	24
7: Description of CASA Parameters	25
8: <i>In vitro</i> Fertilization Protocol	26
9: TSSK1 Kinase Activity Assay	32
10: MBPp and AMARA Consolidated Curve	33
11: Luminescence with Varying TSSK1 Concentrations	34
12: List of Inhibitors and TSSK1 Inhibition Assay	35
13: Effect of Compound C on TSSK1 Activity	36
14: Investigation of AMPK in Murine Sperm	48
15: Effect of Compound C on Sperm Signaling Events	49
16: Computer-Assisted Sperm Analysis on Motility and Hyperactivation of Sperm in the Presence of Compound C	50
17: The Effect of Compound C on the Acrosome Reaction	51
18: <i>In Vitro</i> Fertilization	52

LIST OF ABBREVIATIONS

Ab – antibody	GFP – Green Fluorescent Protein
ADP – Adenosine Diphosphate	hCG – Human Chorionic Gonadotropin
AMPK – 5' Adenosine-Monophosphate activated Kinase	HCO₃⁻ – Bicarbonate
ANOVA – Analysis of Variance	hrTSSK1 – Human Recombinant TSSK1
AR – Acrosome Reaction	HTS – High Throughput Screening
ATP – Adenosine Triphosphate	IgA – Immunoglobulin A
βME – 2-mercaptoethanol	IgG – Immunoglobulin G
BSA – Bovine Serum Albumin	IU – International Units
CAMK – calcium/calmodulin-dependent kinase	IVF – <i>In Vitro</i> Fertilization
Cap – capacitation [conditions]	IP – Immunoprecipitation
CASA – Computer-Assisted Sperm Analysis	kDa – KiloDalton
CC – Compound C	Kenpau – Kenpaullone
DIC – Differential Interface Contrast	MBP – Myelin Basic Protein
DMSO – Dimethyl Sulfoxide	MBPp – Myelin Basic Protein peptide
ECL – Enhanced Chemiluminescence	miRNA – MicroRNA
FACS –Fluorescence-Activated Cell Sorting	mTYH – Modified TYH
FITC-PNA – Fluorescein Isothiocyanate – Peanut (<i>Arachis hypogaea</i>) Agglutinin	MW – Molecular Weight
	NC or Non-cap – non-capacitation [conditions]
	PAGE – Polyacrylamide Gel Electrophoresis
	PBS – Phosphate-Buffered Saline

PF – Paraformaldehyde

PI – Propidium Iodine

PKA – Protein Kinase A

PMSG – Pregnant Mare's Serum Gonadotropin

PVDF – Polyvinylidene difluoride

PY – Phosphotyrosine

RIPA – Radioimmunoprecipitation Assay Buffer

RISUG – Reversible Inhibition of Sperm Under Guidance

RLU – Relative Luminescence Unit

ROI – Region of Interest

Roscov – Roscovitine

rTSSK1 – Recombinant TSSK1

SDS – Sodium dodecyl sulfate

TBS – Tris-Buffered Saline

T-TBS – Tris-Buffered Saline + 0.1% Tween-20

T-PBS – Phosphate-Buffered Saline + 0.1% Tween-20

TSSK# – Testis-Specific Serine Kinase #

TYH – Toyoda-Yokoyama-Hosi [media]

CHAPTER 1

INTRODUCTION AND MATERIALS & METHODS

1.1 Introduction

Since the 1950's, women have been the target demographic for developing contraceptive products, placing the majority of the fertility decisions with the female. However, men play an undeniable role in procreation, and deserve an equal opportunity for contraceptive options.

Nearly 37-50% of pregnancies worldwide are unintended (Mosher, Jones et al. 2012), which demonstrates the current need for additional forms of contraception (Fig. 1). Men only have three options in terms of pregnancy prevention: condoms, withdrawal, or vasectomy. Condoms are easily reversed and 98% effective if used correctly, but has a failure rate of up to 82%, according to the Center for Disease Control. Withdrawal prior to ejaculation is a non-permanent option, but only ~72% effective. The final option is a vasectomy, which is nearly 100% effective, but is a surgical procedure that includes the ligation of the vas deferens, thus preventing ejaculation of spermatozoa. However, this procedure is not easily reversed (Control 2015). Evidently, there is a serious lack of male contraceptive options available; however there are several potential sources for alternative contraceptive development and research. Currently there are several targets being studied worldwide that are potential targets for male contraceptives. One such study is of a physical barrier known as “Reversible inhibition of sperm under guidance (RISUG)” in India, where it was developed, or as Vasalgel in the United States (Lohiya, Alam et al. 2014). This polymer is injected directly into the *vas deferens* and can later be reversed by injecting a dissolving agent. RISUG is currently in Phase III trials in India.

Another area of interest for targets of male contraceptives is focused on hormonal targets; gestagens produced from either testosterone or progesterone derivatives, are being investigated, some having reached clinical trials (Nieschlag 2010, Dorman and Bishai 2012). Other targets are non-hormonal: epididymal proteins, opioids, miRNAs, plant extracts, heat/ultrasound treatment, among others (Garside 2013, Kogan and Wald 2014, Murdoch and Goldberg 2014). In particular, one such area under active investigation is on testis-specific protein kinases, which is the main focus of research in our laboratory.

Because sperm undergoes a number of changes between ejaculation and fertilization, sperm-specific proteins are ideal targets for contraception. When the sperm enters the female reproductive tract, it undergoes a process known as “capacitation.” Capacitation includes the acquisition of hyperactive motility (Yanagimachi 1981, Suarez, Katz et al. 1983) and the ability to experience the acrosome reaction – an exocytotic process that allows penetration of the zona pellucida by the sperm cell (Dan 1952). These activation-type steps and physiological changes to the sperm are required for oocyte fertilization (Chang 1951, Visconti 2009). Figure 3 shows the accepted model on the series of biochemical steps which sperm undergoes during capacitation.

Protein kinases are found throughout humans and animals; their role lies in cell regulation. Typically kinases phosphorylate some aspect of a protein. This phosphorylation allows the protein kinase to orchestrate numerous cellular functions, protein localization, and cell cycle activities. Kinases are protein-specific so they are grouped in families; these families share similar sequences and catalytic domains. One subgroup of kinases are those that phosphorylate serine and threonine residues ((Manning, Whyte et al. 2002) and references therein).

The Testis-Specific Serine Kinases (TSSKs) belong to a family of kinases with a major role proposed in spermatogenesis (Li, Sosnik et al. 2011). This group of serine/threonine kinases is part of the 5' adenosine-monophosphate activated kinase (AMPK) branch in the calcium/calmodulin-dependent kinase (CAMK) group (Fig. 2) and lends itself to potentially be a non-hormonal target for a male contraceptive pill via inhibition of TSSKs (Salicioni 2012). The benefit of these kinases lies within its name: testis-specific. Being specific to the testis allows for research in this particular area without much concern for the effect of a kinase inhibitor on other tissues. It is known that members of the TSSK family are expressed postmeiotically in mature male germ cells (Hao, Jha et al. 2004, Li, Sosnik et al. 2011). It has been shown that TSSK1 is localized to the head, specifically the acrosomal region, of the mature sperm, as well as the flagellum (Li, Sosnik et al. 2011) (Fig. 4). Lastly, It is also known that the TSSK1/2 knockout (Xu, Hao et al. 2008, Shang, Baarends et al. 2010) and the TSSK6 knockout (Spiridonov, Wong et al. 2005) phenotypes render male mice infertile (Xu, Hao et al. 2008, Shang, Baarends et al. 2010), suggesting that the TSSK family plays a major role in male fertility and could be a target for specific inhibitors leading to a male contraceptive pill (Li, Sosnik et al. 2011).

One of our lab's research goals is to find a specific inhibitor of TSSKs that would suppress their activity to a level that would render the kinase ineffective. Knowledge of TSSKs' position on the murine and human kinome led to the notion that an AMPK inhibitor may be suitable for the role of suppressing TSSK activity. AMPK is a heterotrimeric protein kinase comprised of three subunits: α , β , and γ , with α being the catalytic subunit (Hardie 2015). As discussed previously, TSSKs are found on the AMPK

branch of the kinome. AMPK acts as an ATP sensor within the cell, maintaining energy balance via stimulation of catabolic pathways and inhibition of anabolic pathways (Liu, Chhipa et al. 2014, Nguyen, Alves et al. 2014). There exists an AMPK inhibitor, Compound C (CC), –also known as “dorsomorphin” and/or “BML-275”– that is selective, and is a competitive inhibitor at ATP-binding sites (Nguyen, Alves et al. 2014). Based on the literature, especially Nguyen et al (2014) and de Llera et al (2012) (Hurtado de Llera, Martin-Hidalgo et al. 2012), it has been proposed that CC has a significant effect on sperm in a number of other species, including humans.

Our research looks into the biochemical regulation of mouse TSSKs and its role in the capacitation process and therefore, their potential for a non-hormonal target for contraception. By using a variety of experimental conditions, the objective of our current investigation is to find a selective inhibitor of TSSKs, using TSSK1 as a model kinase, which can be used in an easily reversible, oral contraceptive for men, thus providing another option for men to choose from when considering contraceptive alternatives.

1.2 Materials & Methods

1.2.1 Aim 1 Materials

1.2.1.1 ADP-Glo™ kit

The ADP-Glo™ kit was purchased from Promega Corporation (Madison, WI) and includes Ultra Pure ATP and ADP, Glo reagent, and the detection agent (luciferase/luciferin). 96- and 384-well plates were purchased from VWR Scientific (Radnor, PA). All reactions were performed in a kinase buffer consisting of a Tris-HCl mixture (Trizma base from Sigma-Aldrich, St. Louis, MO and HCl from Fisher,

Waltham, MA), at a pH of 7.4, MgCl₂ (Sigma-Aldrich), bovine serum albumin (BSA) from Sigma-Aldrich, and MilliQ water.

1.2.1.2 Kinases and Substrates

Human recombinant TSSK1 was purchased through Invitrogen (Carlsbad, CA) and was the kinase used for all kinase assays. Two substrates were used for the standardization of the kinase assay: myelin basic protein peptide (MBPp) and AMARA. MBPp was purchased through Sigma-Aldrich, and the AMARA substrate was purchased via AnaSpec, Inc., (Fremont, CA).

1.2.1.3 Inhibitors

To observe TSSK1 inhibition, several inhibitors were used: Staurosporine, H7, NMPP1, Roscovitine, Kenpaullone, SU6656, H89, SKI 606, and Compound C. Staurosporine and Roscovitine were purchased from LC Labs (Woburn, MA). NMPP1, Kenpaullone, H89, and SKI 606 were purchased from Cayman Chemical (Ann Arbor, MI). H7 was purchased from Tocris Bioscience (Bristol, UK). SU6656 was purchased from Sigma-Aldrich. Compound C was purchased from Enzo Life Sciences (Farmingdale, NY). DMSO was required for preparation of stock solutions (typically 10mM) of several of these inhibitors, including Compound C; thus DMSO (at the corresponding percentage in final solutions) was used as the vehicle control.

1.2.1.4 Equipment and Data Analysis

Incubation of kinase reactions was performed in an Eppendorf Thermomixer® R at 37°C. Following completion of the assay, the plate was read in a POLARstar Omega plate reader by BMG Labtech. The POLARstar machine was set to read plates at an

interval of 0.10 seconds at a gain of 2500-3600 at 80%. Data was analyzed using both Microsoft Excel and SigmaPlot (by Systat Software Inc.).

1.2.2 Aim 1 Methods

1.2.2.1 Kinase Assay

The initial step in running the assay was preparing the buffer; to do so, Tris-HCl at 40mM and pH of 7.4 was combined with 20mM MgCl₂, BSA at 0.1mg/ml, and MilliQ water. This buffer was used throughout the assay to prepare the other reagents. The ATP was prepared at varying concentrations between 0-1mM using the buffer to dilute the ATP as needed. The substrates (AMARA and MBPp) were prepared at concentrations ranging from 0-100μM using buffer as a reconstitution agent. The kinase (hrTSSK1) was prepared at different concentrations ranging from 0-300ng in the previously described buffer. In some cases, the experimental reagent was prepared by using serial dilution.

In the 96-well plate assays, we decided to have a total quantity of 25μl per kinase reaction. In 384-well plate assays, only a total of 5μl of reagents were included in each well.

When conducting an assay, the reagents were prepared and put into the appropriately sized well plate at a ratio of 1:1:1:2 (buffer, ATP, substrate, kinase, respectively, and +/- inhibitor), and incubated at 37°C for 30 minutes in the Eppendorf Thermomixer® R. Next, the Glo reagent was added; the ratio was 1:1:2, indicating amounts for the reaction, Glo reagent, and detection agent, respectively. For a 96-well plate reaction that carried a volume of 25μl of kinase reaction, 25μl of Glo reagent was added to the reaction following the incubation period. The reaction was allowed to proceed at room temperature for 40 minutes. After this second incubation, 50μl of

detection reagent was added to each reaction and incubated away from light for 60 minutes at room temperature. The plate was then read in the POLARstar Omega plate reader, which generated the number of relative luminescence units (RLUs), a direct indicator of ADP production and, thus, of kinase activity. Figure 5 provides a principle of the ADP-Glo™ kinase reaction and detection, and a summary of the reactions that occurred during this process.

1.2.3 Aim 2 Materials

1.2.3.1 Animals

CD1 retired male breeder mice were purchased from Charles River Laboratories (Wilmington, MA). Transgenic *Acr-GFP* male and female mice were donated from Dr. G. Gerton (UPenn, Philadelphia, PA), who had originally obtained them from M. Okabe (Okabe, Ikawa et al. 1997, Ikawa, Yamada et al. 1999, Nakanishi, Ikawa et al. 1999, Ventela, Mulari et al. 2000, Hasuwa, Muro et al. 2010). All mice were maintained and housed for up to two weeks in a monitored facility at the University of Massachusetts, Amherst; all animal protocols were reviewed and approved by the University of Massachusetts Institutional Animal Care and Use Committee.

1.2.3.2 Media and Inhibitors

Protein extraction of mouse sperm cells was performed in modified Toyoda–Yokoyama–Hosi (mTYH) media (Tateno, Krapf et al. 2013) prepared and stored at 4°C within one month of use. The media was prepared with the following components (in millimolar concentrations, from Sigma-Aldrich): 119.37 NaCl, 4.7 KCl, 1.71 CaCl₂•2H₂O, 1.2 KH₂PO₄, 1.2 MgSO₄•7H₂O, 20 Hepes, 5.56 glucose, 0.51 sodium pyruvate, 10µg/mL gentamicin, and 0.0006% phenol red. To prepare capacitating (Cap)

media using mTYH, 10mg/ml of BSA (Sigma-Aldrich) and 30mM of HCO_3^- (Sigma-Aldrich) was added to the media on the day of the assay. Noncapacitating (NC) media did not have the BSA or HCO_3^- additives. The pH of the media was adjusted using 200mg/mL NaOH (Sigma-Aldrich) to pH 7.2-7.4 (typically falling between 7.32-7.38). M2 media was purchased from Sigma-Aldrich and used during IVF.

The AMPK inhibitor Compound C was prepared in DMSO at 10mM from Enzo Life Sciences (Farmingdale, NY) and was used during subsequent capacitation experiments.

1.2.3.3 Western Blot and Immunoprecipitation Assay

Reagents used in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and the protein molecular weight marker were purchased from Bio-Rad (Hercules, CA). The polyvinylidene difluoride (PVDF) membrane was purchased from EMD Millipore (Billerica, MA). BioMax Light film was purchased from Carestream Health Care, Inc./Kodak, Rochester, NY. Fish skin gelatin was purchased from Sigma-Aldrich. Antibodies were purchased as follows: AMPK α poly- and monoclonal, and anti-PKA from Cell Signaling (Danvers, MA), monoclonal anti-PY from EMD Millipore (Billerica, MA), and anti-tubulin clone E7 from DSHB. Secondary antibodies (anti-mouse IgG, anti-rabbit IgG, HRP and High + Light chain) were purchased from Jackson ImmunoResearch (West Grove, PA). The inhibitors used to create RIPA+ and PBS+ included NaVO_4 (1mM from Sigma), β GP (40mM from Sigma), and 50X protease inhibitor from Roche.

C2C12 murine lysates were purchased from Cell Signaling. 293T cells were grown in-lab in a monolayer and pelleted prior to lysis. Magnetic A-beads and G-beads were purchased from Millipore (Billerica, MA).

1.2.3.4 Sperm Cell Viability Assay

The Live/Dead® Sperm Viability Kit was purchased from Life Technologies (Carlsbad, CA) and was prepared as indicated in the manufacturer's protocol. A HEPES-buffered saline solution was prepared and contained: 10mM HEPES (from Fisher), 150mM NaCl (from Sigma), and 10% BSA at a pH of 7.4.

1.2.3.5 Computer-Assisted Sperm Analysis (CASA)

Mouse sperm cells were loaded into a single side of a 2-chambered 100-micron slide (Leja, Netherlands). The slide was read using an Olympus CX41 microscope, and sperm motility data captured by HTR motility software (Equine – Version 14 Build 008 programed for mouse sperm by Hamilton-Thorne/Pyramid Technical Consultants, Inc.). Data acquisition was programmed at the following settings: Image capture at 60 frames/second at a rate of 60Hz under a 4X objective. The minimum acceptable values were set at 20% motility, minimum contrast at 35, minimum cell size at 4 pixels, progressive cells had to have a path velocity (VAP) of 50.0 μ /s, a straightness of 50%, and of the slow cells, a VAP of 10.0 μ /s, and a straight line velocity (VSL) of 0.0 μ /s. Five fields were captured in each treatment and the results run through the CASAnova analysis software developed by Goodson et al. (2011). The data capture files (.DBT) were imported to Microsoft Excel to observe trends in motility and hyperactivity.

1.2.4 Aim 2 Methods

1.2.4.1 Protein Extraction

Mice were sacrificed by CO₂ followed by cervical dislocation, and the cauda epididymides removed and cut to allow mouse sperm cells to swim out in NC media for ten minutes at 37°C. Following swim out, the remaining epididymis tissue debris was removed from the 2ml-eppendorf (Radnor, PA), and sperm cell suspension split amongst 1.5ml-eppendorf tubes for further treatments if necessary. Cells were then centrifuged for two minutes at 12500rpm; the supernatant was discarded, leaving the pellet. To wash the cells and prevent further protein degradation, cold 1X filter sterilized PBS was added to the pellet. The cells were then centrifuged at 13,400rpm for three minutes to re-pellet them. The supernatant was discarded and the tubes placed on ice immediately. For cell lysis, 5X sample buffer was added to the cell pellets. The tubes containing the cell lysates were boiled for four minutes and centrifuged again for five minutes at 13,400rpm. The supernatant contained the protein and was transferred to a new tube and saved at -80°C for future use. For analysis of signaling events by Western Blotting of PY or PKAS, sperm cell lysates stored at -80°C were used within 24-48h after sample collection.

1.2.4.2 Sperm Capacitation

Following swim out in NC media, sperm was allocated into individual treatment tubes and exposed to the previously described Cap media for one hour at 37°C. Typically, the treatments were as follows: NC, Cap, Cap + DMSO (at 1%), Cap + CC @ 0.1μM, 1μM, 10μM, 30μM, 50μM, and 100μM. For appropriate exposure to this inhibitor, cells were pre-incubated in the NC media with the inhibitor for 15 minutes prior to the introduction of the Cap media.

1.2.4.3 Compound C

When testing for the effect of CC on sperm, cells were pre-incubated in NC media and varying concentrations of CC for 15 minutes prior to the addition of Cap media. Following the addition of Cap media, cells were incubated for an hour to induce sperm capacitation. Samples were then either preserved via protein extraction, or used for further experimentation.

1.2.4.4 Western Blot

The protein extracts were thawed, 2-mercaptoethanol (5% β ME, from Bio-Rad) was added to each extract (at 5% of total volume), and boiled at 100°C for three minutes. Samples were then centrifuged for a short spin at maximum speed and loaded into a polyacrylamide gel. The gel percentage varied depending upon the type of initial primary antibody to be applied: AMPK required a 10% gel, and PKA required an 8% gel. An electrophoresis was performed and the proteins were transferred to 0.45 μ m PVDF membrane for 90 minutes (Laemmli 1970).

1.2.4.4.1 Anti-PKA substrates

The membrane was blocked with 5% milk (prepared in 1X T-TBS –TBS containing 0.1% Tween-20) for an hour at ambient temperature, then incubated with anti-PKA substrate (rabbit monoclonal, Cell Signaling) at 1:10,000 prepared in 5% BSA/T-TBS 1X overnight at 4°C. The following day, the membrane was washed with 1X T-TBS and then incubated with anti-rabbit-HRP (GE Healthcare) at 1:10,000 in 5% milk/T-TBS 1X for two hours at room temperature. Again, the membrane was washed and finally the membrane was developed using ECL Prime (GE Healthcare) and BioMax Light Film.

1.2.4.4.2 Anti-PY

Following exposure to PKAs, membranes were stripped and re-probed with another antibody. Stripping solution comprised of 62.5mM Tris-Cl (pH 6.5) and 2% SDS was combined with β ME (0.74% of total volume) and poured on the membrane. The membrane was incubated at approximately 59°C for 15-30 minutes on a rocker. After discarding the solution, the blot was rinsed with MilliQ water ten times and then washed for longer increments with 1X T-TBS to rid the membrane of β ME.

To probe with a different primary antibody, the membrane was blocked again; when using anti-phosphotyrosine (α PY), the membrane was blocked with 20% fish skin gelatin (Sigma-Aldrich) for one hour at room temperature, incubated with α PY at 1:10,000 in T-PBS 1X (PBS plus 0.1% Tween-20) overnight at 4°C, then washed with T-PBS 1X, incubated with anti-mouse-HRP at 1:10,000 prepared in T-PBS 1X for one hour at room temperature, washed with 1X T-PBS, and lastly, developed using ECL.

1.2.4.4.3 Tubulin

To blot for tubulin antibody, following stripping, the membrane was blocked with 5% milk in 1X T-TBS for one hour at ambient temperature. The primary antibody was monoclonal E7 at either 1:1000 or 1:5000 in 1% milk in T-TBS 1X overnight at 4°C, the membrane was washed the next day, then incubated for one hour at room temperature with anti-mouse-HRP at 1:10,000 in 1% milk in T-TBS 1X. The membrane was washed for an hour and then developed with ECL.

1.2.4.4.4 AMPK α

When probing for AMPK, either a monoclonal (clone F6) or polyclonal anti-AMPK α antibody was used. Typically 2×10^6 mouse sperm cells were loaded per lane.

Following protein transfer onto a PDVF membrane, the blot was blocked with 5% non-fat milk in T-TBS for one hour at room temperature. The primary antibody was suspended in a 1% milk solution at a concentration of 1:1000. The subsequent day, the membrane was washed with T-TBS. The secondary antibody was applied –the monoclonal primary antibody required anti-mouse secondary antibody, while the polyclonal primary antibody necessitated anti-rabbit –at 1:10,000 in 1% milk and T-TBS. The membrane was washed for a few short washes in T-TBS and the membrane was developed using ECL.

1.2.4.5 Immunoprecipitation

Sperm samples for immunoprecipitation were prepared in NC media and the entire swim out of sperm cells from one mouse was pelleted and the supernatant discarded. Performed on ice, RIPA+ the inhibitors NaVO₄ (1mM), βGP (40mM), and 50X protease inhibitor was prepared and added directly to the pellet of cells. For cell lysis, the tube with the cells was incubated for 15 minutes on ice and vortexed every five minutes; it was then centrifuged for five minutes at 4°C at 3000rpm.

The supernatant was separated from the pellet. Add 10% SDS to the RIPA+ solution at a concentration of 0.5%. This new solution was added to the remaining pellet, to proceed with a sequential protein extraction for sperm cells. Incubation on ice and vortexing every five minutes was repeated for a total of 15 minutes. It was centrifuged again for five minutes at 4°C at 3000rpm and the supernatant was pooled with the previous supernatant. A pre-determined volume (based on the number of cells desired per well) was removed and put into a separate tube (this was an “input” sample; the other portion was used for immunoprecipitation). To the IP samples, PBS+ inhibitors were added to ensure not more than 0.1% of SDS in final IP reaction; anti-AMPK-alpha was

added at 1:100 (or tubulin (monoclonal E7) at 1:500 in control IP). These tubes were incubated on a rotator at 4°C for 1.5 hours.

During incubation, an equal combination of A-beads and G-beads were prepared via washing with cold PBS + 0.1% Tween-20 (Sigma-Aldrich) and then the supernatant removed and the tubes put on ice. After incubation on the rotator, the tubes were spun down and the supernatant, containing the AMPK-Ab complexes, transferred to the tubes containing the beads, and the reaction was mixed. The tubes were put back into the cold rotator for an additional two hours.

Using a magnetic rack, the beads were washed three times and the supernatant discarded. New tubes were prepared with β ME while 2X sample buffer was added to the beads and vortexed. Tubes were then boiled for ten minutes, vortexed, and spun down at 13,400rpm for one minute. The supernatant was removed and put into the tubes containing β ME, leaving the beads behind. These samples were stored at -20°C until their use the next day.

1.2.4.6 CASA

After the hour of capacitation (as described above), 20 μ l of sample (equivalent to approximately $2-4 \times 10^5$ cells) were removed from the middle of each tube and put into a single slide's chamber. CASA/CASAnova then analyzed samples.

1.2.4.7 Acrosome Reaction

After an hour of incubation in capacitating medium, the calcium-ionophore (A23187) (Sigma-Aldrich) was added at a concentration of 10 μ M/reaction. Samples were incubated for another 30 minutes at 37°C. Parallel samples without A23187 were processed for assessment of spontaneous AR. In addition, samples were collected after a

very brief incubation of sperm samples in Cap medium (considered time zero, T0) and taken as AR controls.

1.2.4.8 PNA Fluorescence Imaging

Following the induction of the acrosome reaction, one milliliter of 4% paraformaldehyde (PF) (Fisher, Framingham, MA) was added to each treatment and samples incubated for ten minutes at room temperature to fix the cells in solution. To remove the PF, tubes containing the samples were centrifuged at 2,500rpm for five minutes, the supernatant discarded, a milliliter of 1X PBS added, centrifuged again at 2,500rpm for five minutes, the supernatant discarded, and the pellet resuspended in 200 μ l 1X PBS.

Using a wide-bore pipet tip (VWR), a 25 μ l droplet of each sample was put onto clean slides (Gold Seal Rite-On Micro Slides, Portsmouth, NH) and allowed to air-dry for 5-10 minutes. To permeabilize the cells, 20 μ l of 100% Methanol (Fisher) was added to each droplet for 30 seconds then aspirated. To ensure the remaining methanol was removed, the slides were washed with 20 μ l of 1X PBS for one minute each, then the PBS was aspirated; this was performed three times. To detect the cell's acrosomal region, 20 μ l of FITC-PNA (prepared at 1:50 from stock, from Molecular Probes Eugene, OR) was applied to each treatment; the slides were then incubated for 30 minutes in a dark chamber.

Again, the cells were exposed to a series of washes and aspirations with 1X PBS. A mounting media comprised of glycerol:PBS (9:1) (glycerol from Fisher) was applied to each treatment (4 μ l) and a clean coverslip (Corning, No. 1.5 from Tewksbury, MA) was applied to each slide. Using a fine pair of forceps, the coverslip was lightly pressed into

the slide to ensure even coverage of the mounting media throughout the treatment. The slides were then sealed with nail polish and stored at 4°C in a dark chamber. Cell counting and AR assessment was performed within 24h.

Slides were read and visually quantified via differential interface contrast (DIC) and fluorescent microscopy using a Nikon Eclipse TE300 and a 60X objective. The camera was an Andor/Zyla and the software used was NIS Elements.

1.2.4.9 Viability

To monitor the viability of sperm cells, especially following the addition of CC, the Life Technologies Live/Dead® Sperm Viability kit was utilized. Reagents were prepared as indicated in the protocol. Following capacitation, a small volume from each treated sample was put into a separate tube (approximately $1-2 \times 10^6$ sperm cells were removed per sample for viability testing). The prepared SYBR 14 (in kit) was added to the new tube at 100nM and incubated for five minutes at 37°C; the propidium iodine (PI) was added at 12μM and incubated at 37°C for an additional five minutes. To wash away the excess PI, the tubes were centrifuged for five minutes at 2,500rpm and the supernatant discarded; 500μl of HEPES buffer was added to each tube. The samples were centrifuged again at the same speed, and for the same duration, and the supernatant was discarded. Pellets were resuspended in approximately 200μl of HEPES buffer and were mounted to slides and visually quantified using the same technique described in the fluorescent imaging section (paragraph 1.2.3.2.8). By staining with two unique reagents, the viability status of each cell was determined. SYBR 14 is a small, membrane-permeable molecule that can enter sperm cell nuclei and produce a green signal under fluorescent imaging. PI is excluded from viable cells and is a known membrane-

impermeant dye; however, when a cell dies or its structure is seriously damaged, their nuclear pores expand, allowing for PI to enter. A red signal is emitted under fluorescent microscopy (Garner and Johnson 1995).

1.2.4.10 *In Vitro* Fertilization (IVF)

In vitro fertilization was performed over 4-5 days beginning by injecting the female mice intra-abdominally with 5IU with pregnant mare's serum gonadotropin (PMSG from Sigma) (Day 1) to induce superovulation. Thirteen hours prior to sacrifice (evening of Day 3), females were injected intra-abdominally with human chorionic gonadotropin (hCG, from Sigma); IVF media was also prepared on Day 3. Capacitating TYH media was prepared and 500 μ l of media was put into well plates (Thermo Scientific nunclon surface). The well plate and remaining Cap media were placed in a 37°C CO₂ incubator (5.1% CO₂) with a loose lid for appropriate O₂/CO₂ exchange.

The following morning (Day 4), a single male mouse was sacrificed and the sperm was allowed to swim out in NC media for ten minutes. The sperm was pre-incubated with the CC (30 μ M or 100 μ M) or H89 (50 μ M) inhibitors followed centrifugation at 2,500rpm for five minutes. The supernatant was discarded and the pellet was resuspended in Cap media. The remaining inhibitor was added to bring it up to the appropriate concentration and the whole sample was transferred to a 2-ml tube. Capacitation was allowed to occur over the next hour and a half in the CO₂ incubator.

While the sperm was undergoing capacitation, the female mice were sacrificed. The reproductive tracts of the females were removed and put into warmed M2 media. Using a fine pair of forceps, the oviducts were punctured to release the cumulus/oocyte complex. The complex was carefully transferred to a separate, fresh dish of M2 media

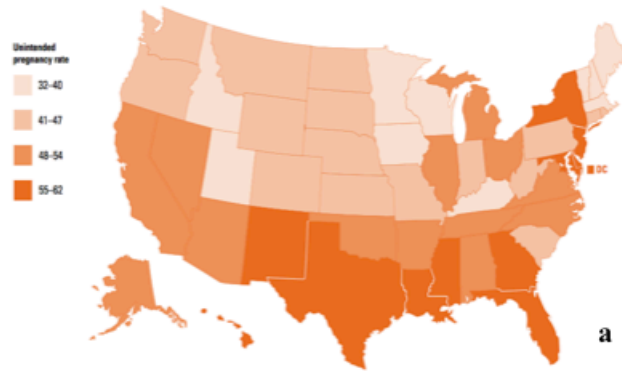
using a glass mouth pipet in order to separate the complex from the reproductive tract. The oocytes were then transferred to the IVF well plates.

When the sperm was capacitated, 10 μ l of sperm was added to the well plates containing the oocytes. Inhibitors were added to the wells (if necessary) and the entire plate was incubated for four hours in the CO₂ incubator. After that time period, oocytes were washed and returned to the incubator for approximately 20 hours. The next day (Day 5), oocytes were observed under the Nikon microscope at 20X and 60X magnification. Oocytes were quantified as 2-cell, 1-cell, or degraded embryos. IVF was also double-checked on Day 5.

1.2.4.11 Statistical Analysis

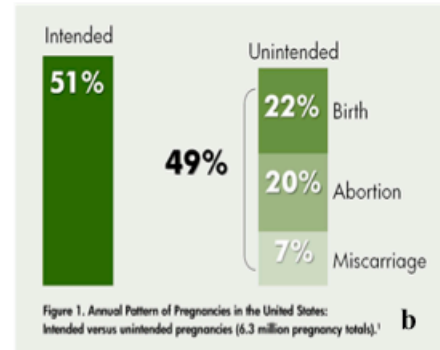
For all quantified data in Chapter 3, Analysis of Variance (ANOVA) was used for statistical analysis. In most cases, the data (percentages) were transformed to comply with the assumptions necessary for ANOVA analysis. Post-test comparisons were made using the Tukey-Kramer test or Fisher test to determine p-values.

Unintended Pregnancy Rates, by State, in 2010



*Rates for Arizona, Indiana, Kansas, Montana, Nevada, New Hampshire, North Dakota and South Dakota estimated by multivariate regression.

Kost, 2015



Association of Reproductive Health Professionals, 2011

Men	Women ^c
Condoms	Hormone-based methods: Pills, implant, injection, patch, ring
Withdrawal	Barriers: diaphragm, condoms, spermicides
Vasectomy	IUDs
	Permanent methods: tubal ligation, transcervical sterilization

Figure 1: Unintended Pregnancy and Contraceptive Options. a) Rate of unintended pregnancy in the United States (2010) (by Kost et al., 2015). b) The percentage break down of unintended pregnancies in the U.S. by outcome. c) Contraceptive options currently available to both men and women. From the table, it becomes apparent that women have a greater number of choices than men.

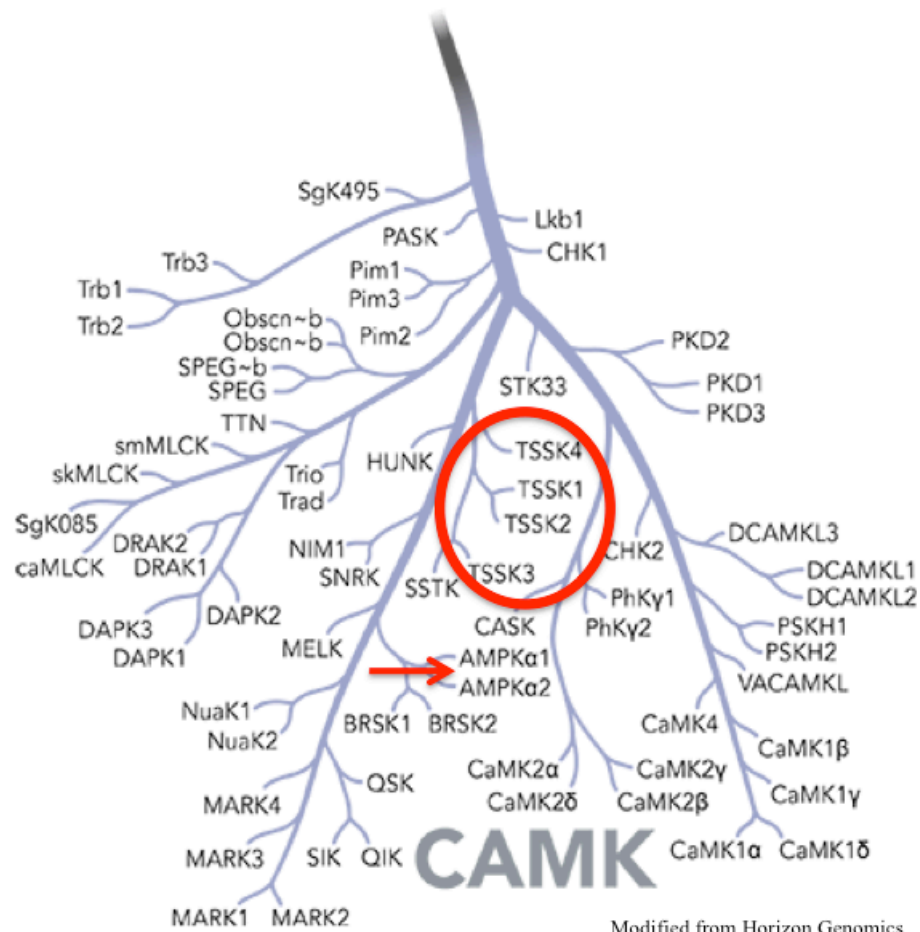
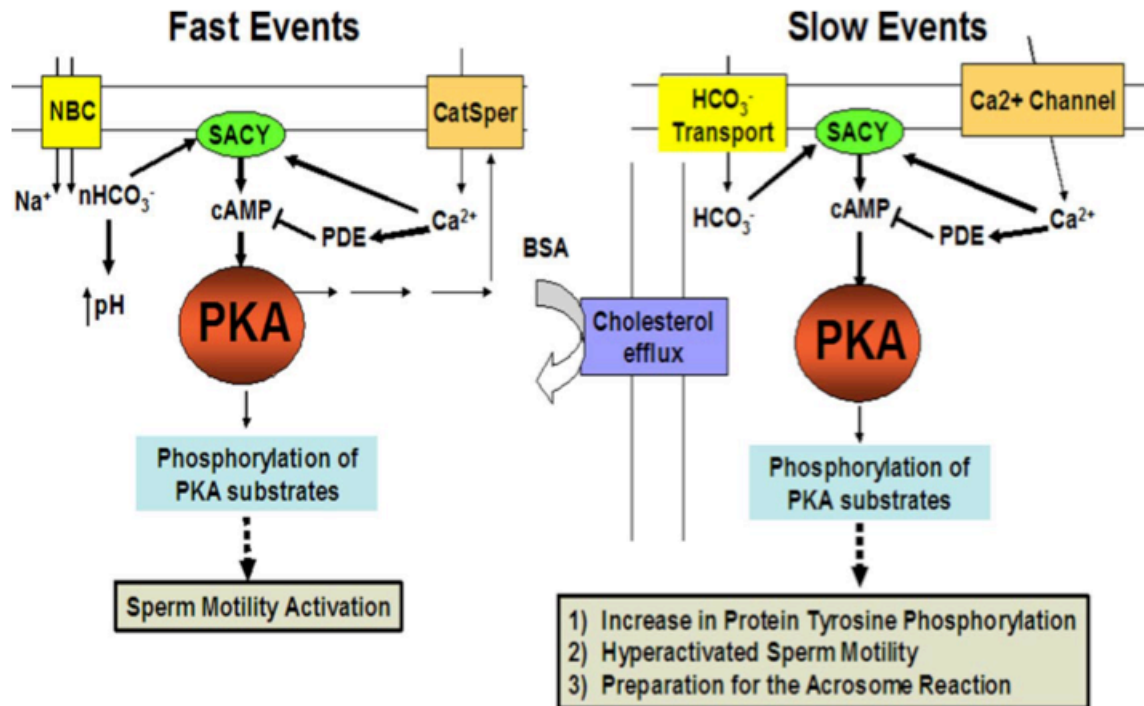


Figure 2: CAMK Branch of the Human Kinome. The CAMK branch of the human kinome; the TSSK family within the AMPK superfamily is highlighted.



Visconti, 2009

Figure 3: Biochemical Events During Sperm Capacitation. Two major events have been proposed to occur during sperm capacitation: fast events which take place within the first few minutes of exposure to capacitating conditions; and slow events which typically occur after 45-60 minutes. Over time (called "capacitation"), several steps are known to occur, including, an intracellular increase of three key molecules: calcium, bicarbonate, and hydrogen peroxide. These three increases cause an activation of adenylyl cyclase which then produces cyclic AMP (cAMP). cAMP activates protein kinase A (PKA). PKA is known to phosphorylate numerous proteins and it has been shown that PKA activity increases during mouse sperm capacitation. Additionally, it has been suggested that cAMP/PKA may play a role in protein tyrosine phosphorylation in several species including humans (Visconti, 1995 a,b; Aitken, 1995; Leclerc, 1997; de Lamirande, 1997).

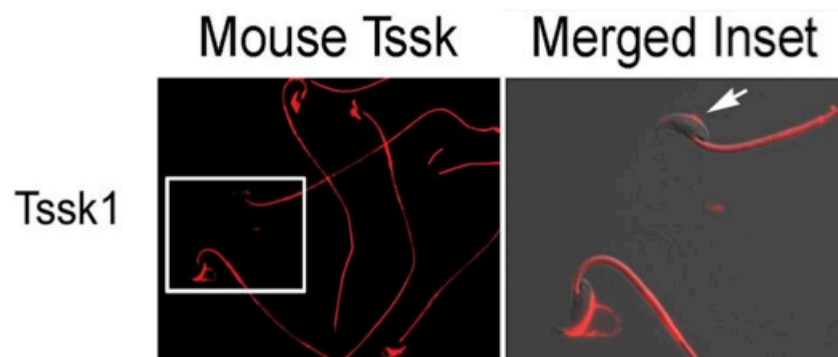


Figure 4: Immunolocalization of TSSK1. Tssk1 has been found localized in both the acrosomal region and the flagellum of mouse sperm and human sperm (Li et al., 2011). Here, fluorescence imaging depicts mouse TSSK1 immunolocalization (psuedocolored in red); the arrow indicates an acrosome-reacted sperm cell.

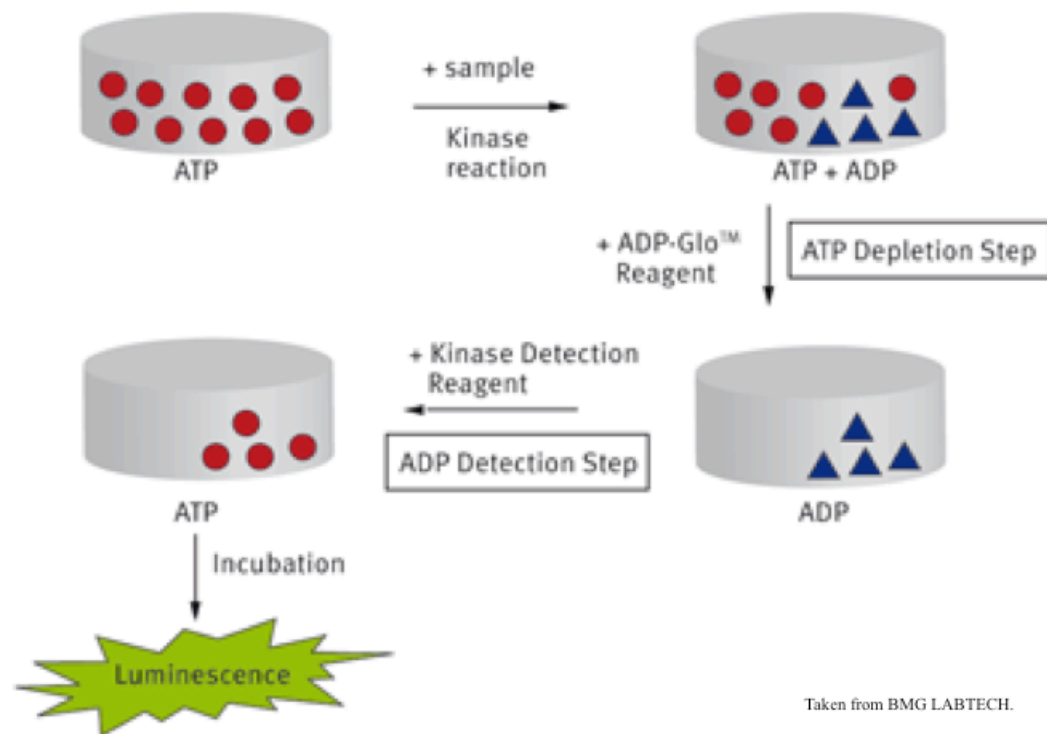


Figure 5: ADP-Glo™ Mechanism. The methodology of the ADP-Glo™: With a starting quantity of ATP, the kinase, substrate, and other starting material (inhibitors, etc.), the kinase phosphorylates the substrate using ATP and releasing ADP. The addition of the ADP-Glo reagent causes the remaining ATP to be depleted, leaving only ADP. The kinase detection reagent is then added to the plate causing ADP to be converted to ATP. Luminescence is produced using the remaining ATP by coupling it to a luciferin-luciferase reaction; the final output, in relative luminescence units (RLUs), is indicative of kinase activity (Zegzouti et al., 2009).

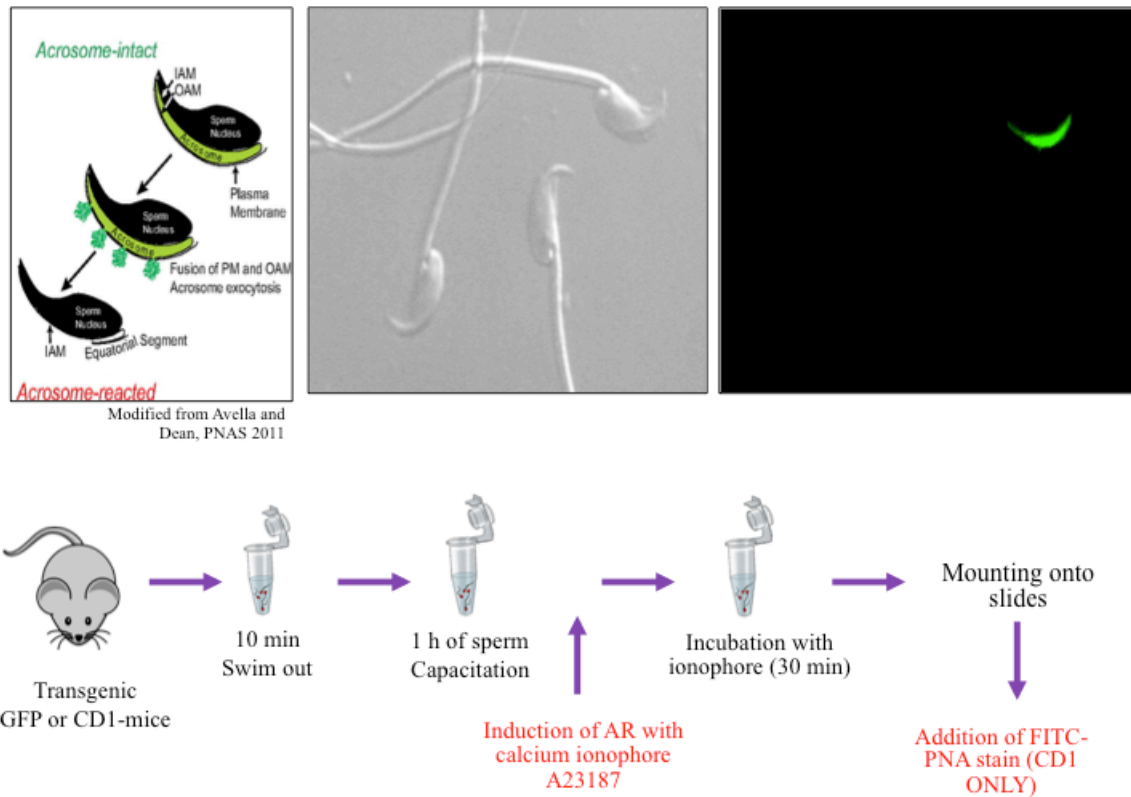
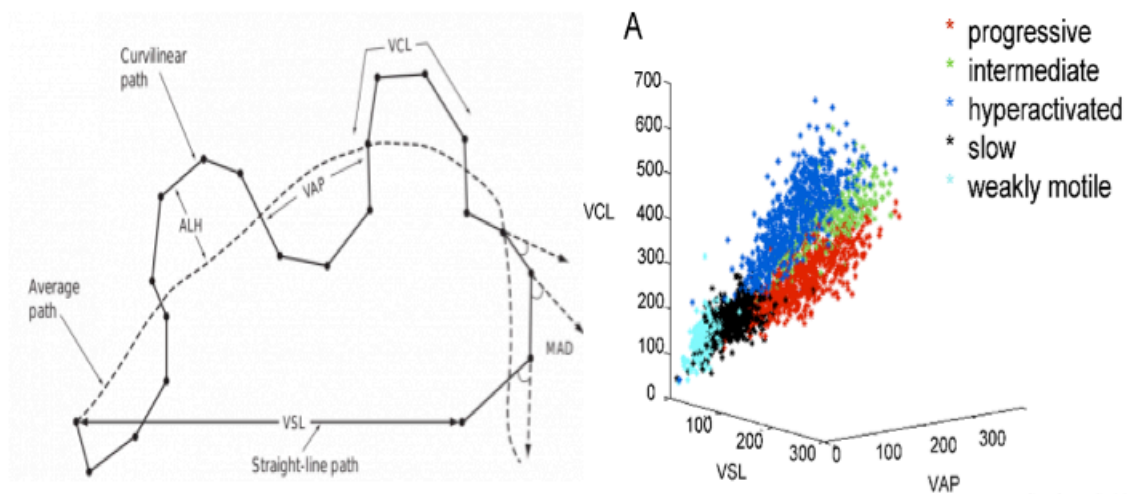


Figure 6: The Acrosome Reaction. The acrosome reaction (AR) is an exocytotic process in which the sperm cell releases its acrosomal content as it reaches the egg's zona pellucida. To induce the acrosome reaction *in vitro* following capacitation, a calcium ionophore is added and incubated with the sperm. To observe acrosome-reacted sperm, following induction of the AR, cells are mounted onto a slide and when using CD1 mice, they are fixed and stained with FITC-PNA. Cells are imaged and acrosome reaction rate is quantified visually via fluorescent microscopy (upper panel). A = 600x. Transgenic *Acr-GFP* mice may also be used for this reaction without the need for FITC-PNA.



Goodson, S. 2011

VAP – Avg. path velocity
VSL – Straight-line velocity
VCL – Curvilinear velocity
ALH – Amplitude of lateral head displacement
BCF – Beat cross frequency

Figure 7: Description of CASA Parameters. On the left, Computer-Assisted Sperm Analysis (CASA) kinematic parameters (World Health Organization, 2010) and the parameters captured (Table). On the right, the sperm classification system developed by Goodson, et. al (2011). Using the kinematic parameters, sperm may be grouped into five cell motility categories: progressive, intermediate, hyperactivated, slow, and weakly motile.

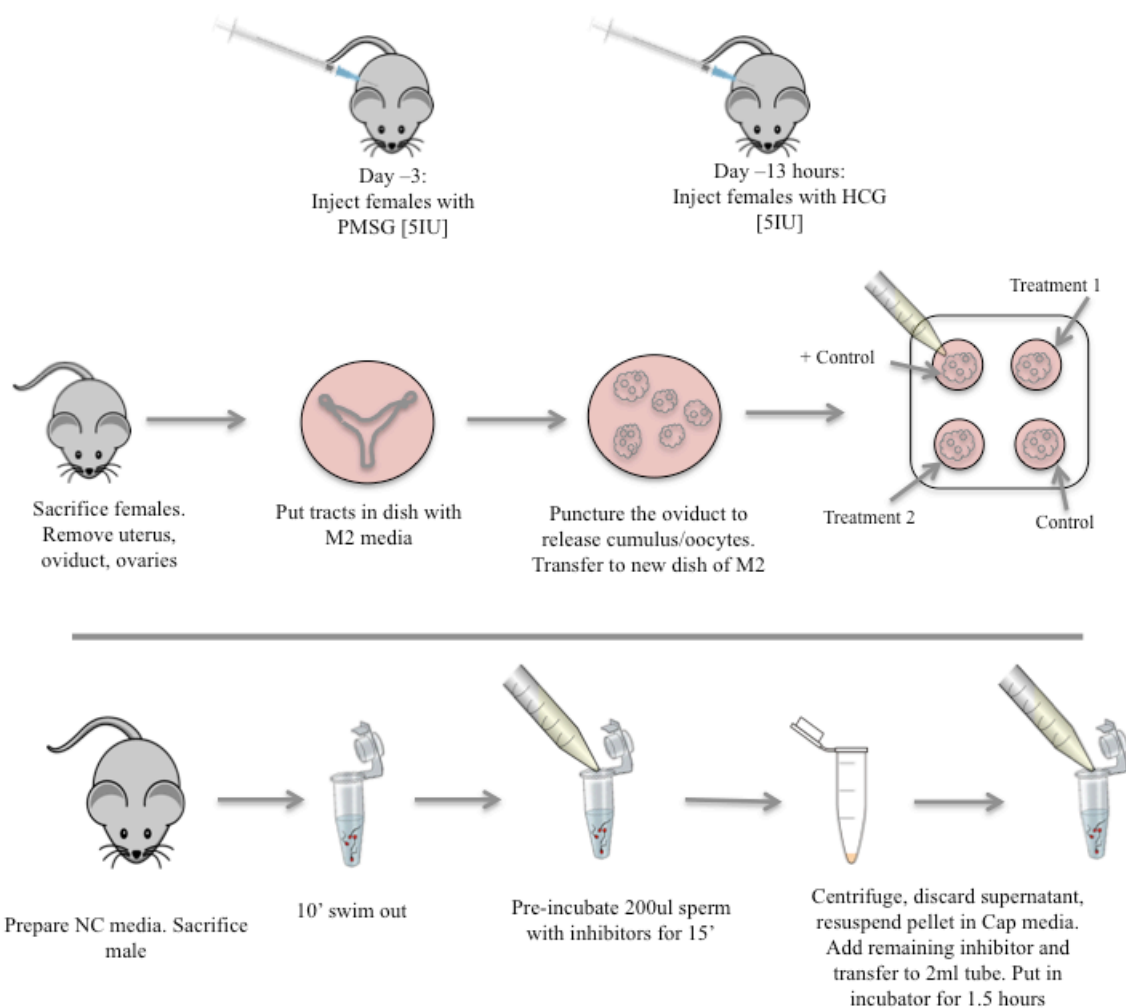


Figure 8: *In Vitro* Fertilization Protocol. The upper panel details the initial steps of an *in vitro* fertilization (IVF) procedure performed using female mice. The lower panel explains the male portion of IVF, the sperm harvesting. Following these two steps, sperm is incubated with the oocytes and the rate of IVF is visually quantified 16-20 hours later.

CHAPTER 2

***IN VITRO* SCREENING FOR SMALL-MOLECULE KINASE INHIBITORS OF TESTIS-SPECIFIC**

SERINE KINASE 1 (TSSK1)

2.1 Rationale

TSSKs are a unique family of serine/threonine kinases localized to the testes; the conserved expression of TSSK genes and well-established role of phosphorylation of sperm proteins suggests that TSSKs have a role in sperm function (Hao, Jha et al. 2004, Li, Sosnik et al. 2011). TSSK1 was the first described member of the TSSK family (Bielke, Blaschke et al. 1994) and it was found in both an active and inactive state. Members of the TSSK family are typically considered “active” when they are phosphorylated; phosphorylation of the hydroxyl group of either the threonine or serine residue within the T-loop using ATP as the phosphate donor activates the kinase. According to *ex vivo* work performed by Bucko-Justyna, there is evidence of TSSK autophosphorylation (Bucko-Justyna, Lipinski et al. 2005, Xu, Hao et al. 2008), which would suggest that the kinase has the ability to self-activate. The goal of our lab is to find a small-molecule inhibitor that is specific to TSSK1. Since our group found TSSK1 to be the most soluble when analyzed in mouse and human sperm samples (Li et al., 2011), and it would prove more amenable to biochemical characterization *in vivo* and *in vitro*, TSSK1 was chosen as the model target for the search of a kinase inhibitor.

In order to progress to high throughput screening (HTS), there are a number of steps necessary to perform prior. Based on the steps outlined by Brooks (Brooks 2012) among others, it is necessary to define reagents and instruments, validate the assay, and optimize conditions before engaging in HTS. The following experiments were designed

to (a) determine assay feasibility, (b) establish experimental parameters, and (c) begin to look at a reduced battery of kinase of potential inhibitors as a method to assess the (d) sensitivity of a homogeneous TSSK1 activity assay in a small scale system, which could then be adapted to an HTS of a larger library of compounds.

2.2 Results

2.2.1 Testing Assay Sensitivity

As shown in Figure 9, the feasibility of using the ADP-Glo™ kit was tested using recombinant purified TSSK1 (rTSSK1). Using the substrate myelin basic protein peptide (MBPp) and ATP, rTSSK1 kinase reactions were performed in a 96-well plate assay to determine whether the ADP-GLO detection system was effective for quantifying TSSK1 kinase activity in vitro. Prior to running the assay, the possibility of TSSK1 autophosphorylation was acknowledged (Bucko-Justyna, Lipinski et al. 2005, Xu, Hao et al. 2008); to investigate the rate of autophosphorylation, rTSSK1 was put through the ADP-Glo™ assay without a substrate, alongside treatments that included substrate –in this case, MBPp. While there was a noticeable amount of autophosphorylation in the rTSSK1 at 25ng and 50ng, what was more significant was that when utilizing as low as 25ng of rTSSK1 in a 96-well plate reaction, rTSSK1 activity could be detected in comparison to reactions containing ATP alone (control). In addition, there was a dose-dependent response to the amount of kinase used; the kinase activity at 50ng of rTSSK1 was nearly double that of 25ng. Of equal importance was that the experiment demonstrated that the assay was functional using this kinase, and kinase activity was detectable at the lower concentration. As a result of this series of experiments, it was determined that rTSSK1 at 25ng could be used for future assays.

2.2.2 Testing Substrate Sensitivity

To continue establishing assay parameters, it was necessary to identify the most appropriate substrate. Our lab had demonstrated that MBPp was a suitable substrate for TSSKs, due to positive published data from radioactive assays utilizing TSSK1 and TSSK2, and also based on studies by others (Bucko-Justyna, Lipinski et al. 2005). However, more recent literature indicated that the peptide, AMARA, could also be a suitable substrate for use with TSSKs (Jaleel, McBride et al. 2005), SIK, and AMPK kinases (TSSKs are members of the AMPK branch of the kinome). To determine which of the two substrates, MBPp or AMARA, was more sensitive to phosphorylation by TSSK, an assay was designed to compare the two substrates (Fig. 10). Both substrates were tested at various concentrations from 0-100 μ M to compare the relative luminescence units (RLU) output. From the resulting graph, significant differences between the RLU outputs of the two substrates can be seen. This experiment indicated that AMARA was more sensitive and likely more specific to rTSSK1, showing a nearly 10-fold increase of RLU output versus MBPp. Further assays were performed using AMARA as the substrate.

2.2.3 rTSSK1 Concentration Curve

Following the previous determinations and because ultimately, the overall endpoint was to bring this assay to HTS, investigation into the suitability and functionality of the assay at lower concentrations was the next step. To do so, ATP and AMARA were both added at 100 μ M to a 384-well plate and rTSSK1 was scaled-down to concentrations ranging from 0-18.25ng per reaction, specifically chosen to lower amounts than previous assays in order to test sensitivity. As shown in Figure 11, we were able

detect changes in TSSK1 kinase activity at concentrations as low as 2.5ng in a 384-well plate format. Until this point, all assays were performed in a 96-well plate with a total reaction volume of 100 μ l; with this new information, we were able to reduce the total reaction volume by using smaller reagent volumes. Subsequent assays were performed in a 384-well plate with a total reaction volume of 25 μ l. The possibility of quantity reduction was necessary for HTS, and this particular experiment encouraged continuation of work with this particular assay on this specific kinase.

2.2.4 Testing rTSSK1 Inhibition *In Vitro*

As stated previously, the goal of this series of experiments was to look for inhibitors of TSSK1. Using the assay parameters established above, an assay was performed that incorporated a battery of known kinase inhibitors, including sperm-specific targets such as PKA or Src. Some inhibitors were broad spectrum, such as Staurosporine that inhibits several protein kinases, whereas others were more specific, inhibiting PKA or AMPK. Listed in the table in Figure 12 are the inhibitors used, listed in order of increasing selectivity: from the most broad-spectrum to the more specific inhibiting reagents. To each reaction, a different inhibitor at 100 μ M or DMSO (control) was added alongside rTSSK1 and AMARA. The corresponding RLU output was graphed in Fig. 12; based on the graph it can be seen that CC and SKI 606 were the most effective compounds to inhibit rTSSK1. Further investigation of CC was performed – because it is an AMPK inhibitor, and TSSKs are found on the AMPK branch, it was hypothesized that CC might be a suitable inhibitor of TSSKs.

Figure 13 depicts the inhibitory effect of CC on rTSSK1 at concentrations from 0.1 μ M-100 μ M. The downward curve indicated a dose-dependent effect of CC on

rTSSK1. Beginning at ~25-30 μ M, there was a significant drop in RLUs, suggesting a more significant inhibitory effect on rTSSK1 activity.

In summary, the abovementioned series of experiments provided a strong rationale and some tools for further experimentation with CC by evaluating sperm functional parameters using and *in vivo* mouse model.

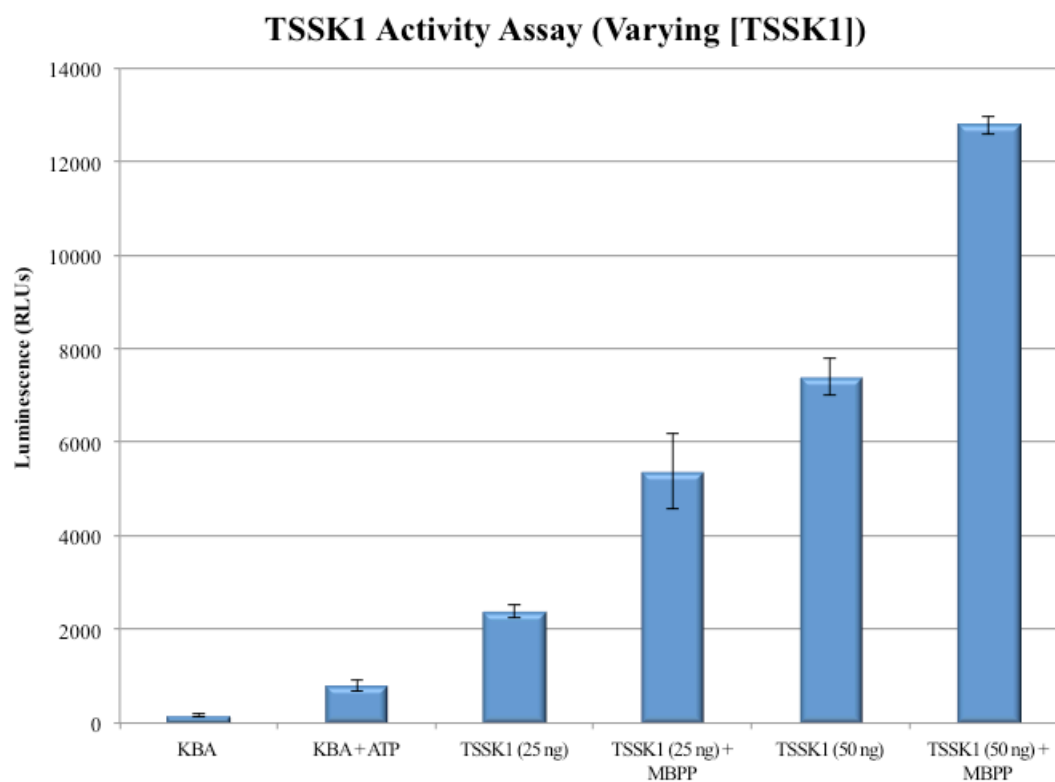


Figure 9: TSSK1 Kinase Activity Assay. To determine the feasibility of the ADP-Glo™ assay utilizing TSSK1, varying concentrations of the kinase were initially tested in the presence or absence of myelin basic protein peptide (MBPp) (100μM) as the kinase substrate.

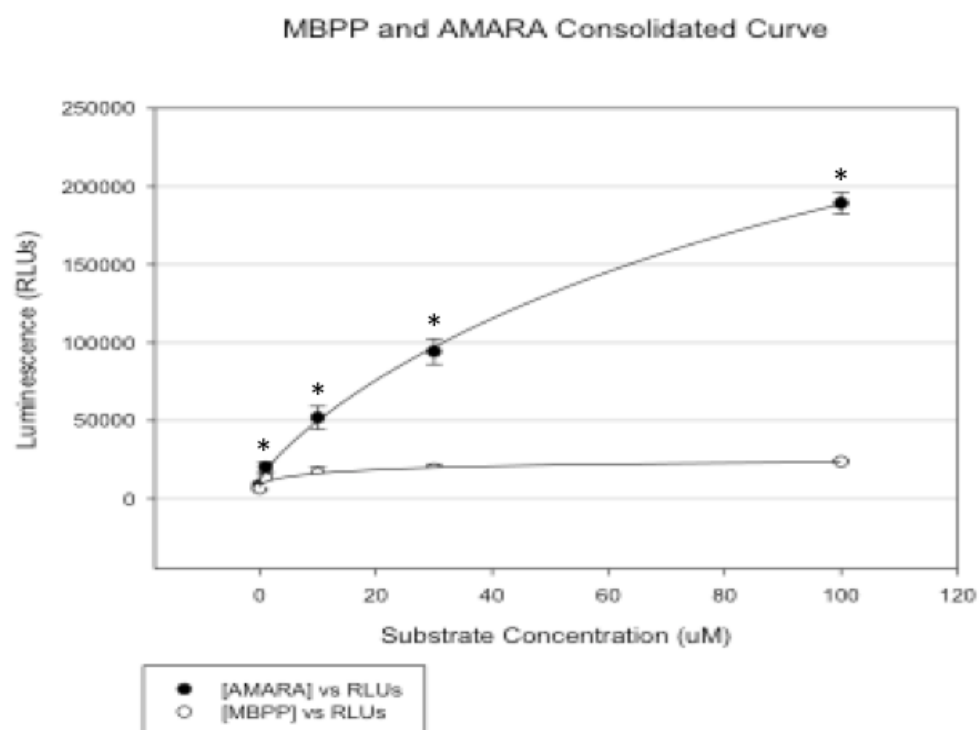


Figure 10: MBPp and AMARA Consolidated Curve. Comparison between two kinase substrates, MBPp and AMARA in terms of ADP produced during a TSSK1 kinase reaction. Based on this higher affinity of TSSK1 for AMARA, further assays were performed using AMARA as the substrate. (*) $p < 0.05$ when compared to MBPp.

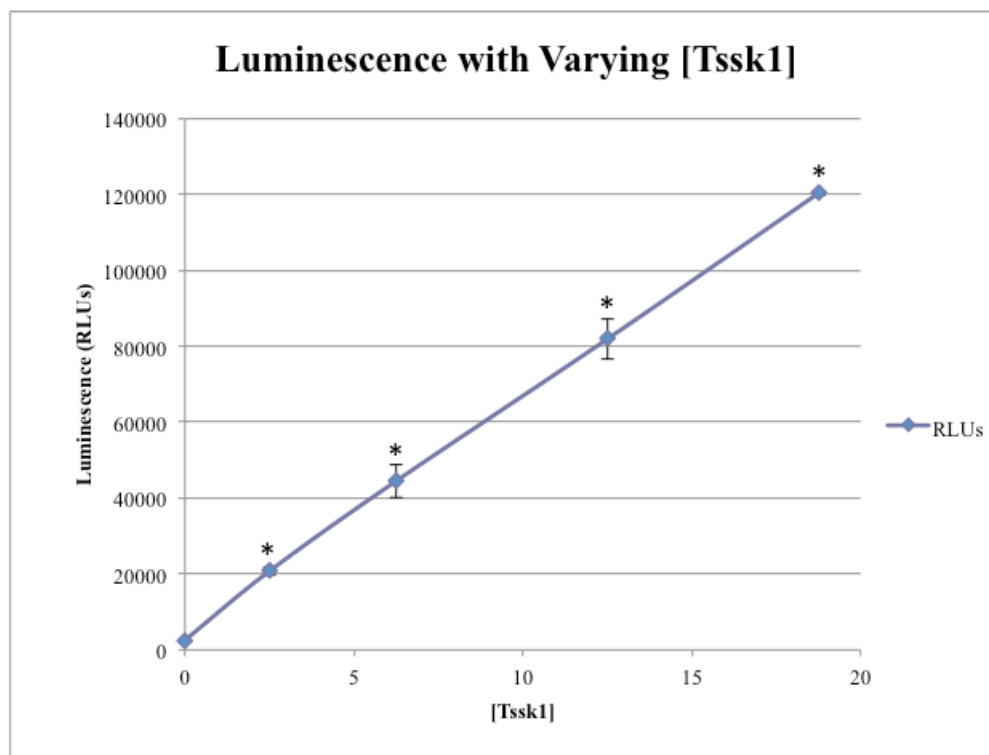


Figure 11: Luminescence with Varying TSSK1 Concentrations. A feasibility test of TSSK1 kinase assay in a 384-well plate format showing that the ADP-Glo™ assay can be used to detect kinase activity at very low amounts of TSSK1 per reaction. (*) $p < 0.05$ when compared to control without TSSK1.

Inhibitors		Broad Specific
Inhibitor	Target	
Staurosporine	Protein kinases	
H7	PKC, PKA	
NMPP1	Mutated protein kinases (Src, tyrosine)	
Roscovitine (ROSCOV)	CDK	
Kenpaullone (KENPAU)	CDK5	
SU6656	Tyrosine kinases	
SKI 606 - Bosutinib	Src, tyrosine kinase inhibitor	
H89	PKA	
Compound C	AMPK	

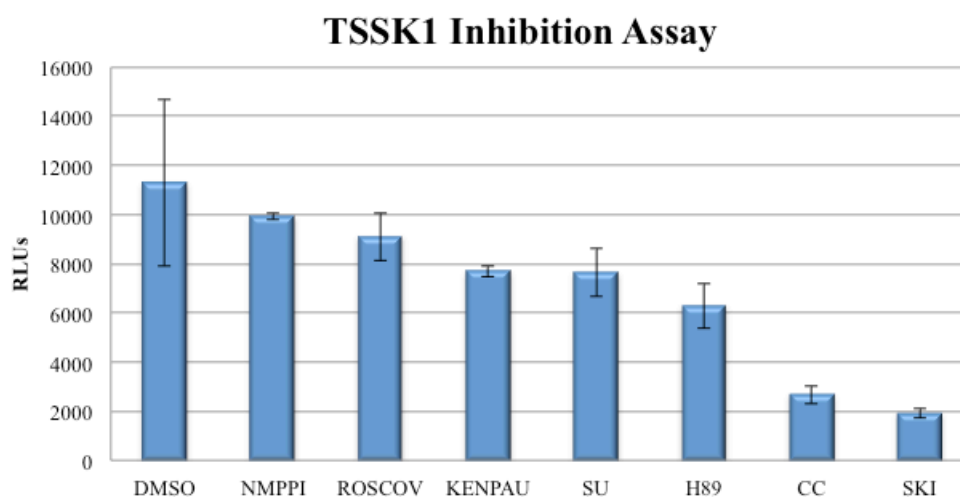


Figure 12: List of Inhibitors and TSSK1 Inhibition Assay. Using the AMARA substrate (containing DMSO) as a control reaction, TSSK1 activity was tested against a battery of kinase inhibitors, listed in the Table (top) based on their relative specificity. Results of the assay indicate that Compound C (CC) and SKI were the most effective inhibitors of TSSK1 activity.

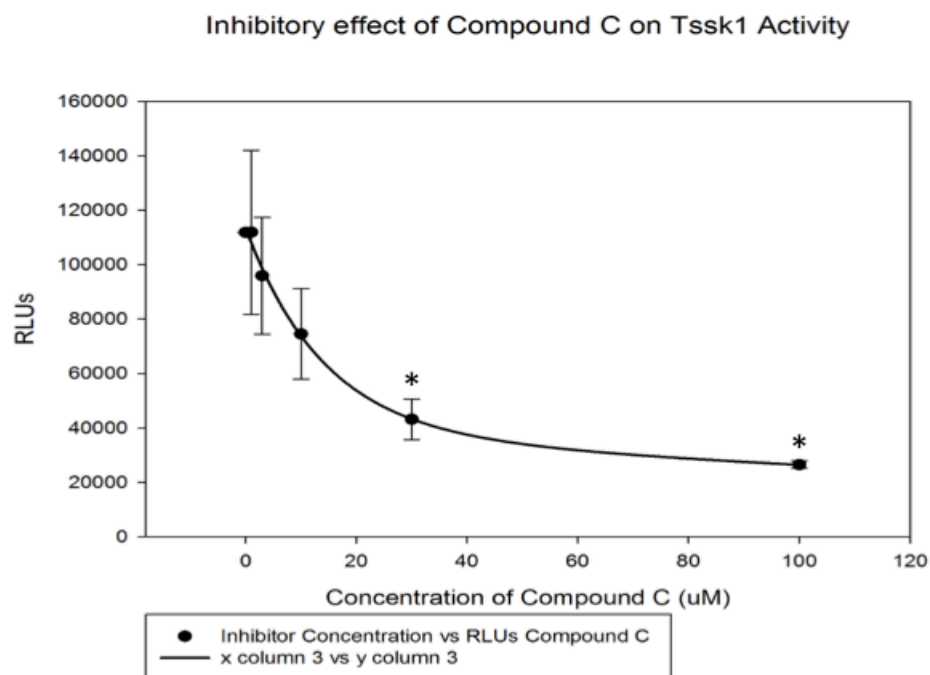


Figure 13: Effect of Compound C on TSSK1 Activity. Compound C (CC) has shown properties as an AMPK inhibitor (Hurtado de Llera et al., 2013; Nguyen et al., 2014) To test its effect on TSSK1, an assay with varying concentrations of CC (from 0.1 μ M-100 μ M) was performed. Data depicted here indicates a dose-dependent inhibitory effect, with significant effects at ~25-30 μ M. (*) denotes $P < 0.05$.

CHAPTER 3

***IN VIVO* TESTING OF SMALL-MOLECULE KINASE INHIBITORS OF AMPK/TSSK**

3.1 Rationale

Following investigation of Compound C (CC) and its inhibitory effect on rTSSK1 activity *in vitro*, the next step was to look at CC in an *in vivo* model system. Based on the proven relevance of these endpoints in evaluating sperm function in mice and other species, we decided to look at the effect of CC on (a) sperm signaling events via phosphorylation; (b) sperm motility; (c) the occurrence of acrosome reaction, and (d) its effect on fertilization ability *in vitro*.

Thus far, there is no clear evidence regarding the role of AMPK in mouse sperm. Due to this information, the findings from Aim 1 of this Thesis (specifically Figures 11 and 12), and the fact that Compound C is a known AMPK inhibitor, we considered it very relevant to (a) determine whether AMPK is in fact present in mouse sperm; and (b) study the effect of a known inhibitor of the AMPK superfamily on mouse sperm function. Among the possible outcomes expected from studies presented in Aim 2 is that AMPK is absent in mouse sperm and thus mouse TSSK1 might be the main kinase target of inhibition by CC.

The importance of studying the effects of CC on PKA and PY are due to the role the two events play in capacitation, and motility. Referring to Figure 3, phosphorylation of PKA substrates is an indication of capacitation (Aitken, Paterson et al. 1995, de Lamirande, Leclerc et al. 1997, Leclerc, de Lamirande et al. 1997, Visconti, Johnson et al. 1997, Breitbart and Naor 1999). We were interested in the effects of CC on capacitation; towards this goal, we investigated PKA substrate phosphorylation when

samples were exposed to CC. While it is not well understood what events occur between PKA substrate phosphorylation and tyrosine phosphorylation (PY), it is known that PY is downstream of PKA in the cellular pathway. In an attempt to orient TSSKs within the pathway, we looked at the effects of CC on both PKA substrates and PY.

As described previously, prior to fertilization the sperm must undergo the acrosome reaction (Dan 1952, Barros, Bedford et al. 1967). It was crucial to investigate the effects of CC on the acrosome reaction, as the AR is critical to successful fertilization. Most of our analysis focused on the effect of CC on sperm spontaneous AR; in some cases, CC effect was also assessed after AR was induced using a calcium ionophore per Wertheimer (and references therein) (Wertheimer, Krapf et al. 2013). A visualization of the methodology used for these experiments can be found in Figure 6. It is worth noting that the Live/Dead® Sperm Viability Kit was consistently run in parallel to ensure that CC was only affecting AR or motility, not viability (data not shown).

In order for a sperm to reach and then fertilize an egg, it must be not only motile, but must have reached a hyperactivated state. First suggested by Yanagimachi, sperm experience a change in motility before fertilization of the oocyte, termed hyperactivation (Yanagimachi 1981, Suarez, Katz et al. 1983, de Lamirande, Leclerc et al. 1997).

Hyperactivation is characterized by an increase in rapid movement creating a frantic, increased amplitude and beat frequency of flagellar waves creating a whiplash motion, and increased lateral head displacement (Mortimer and Mortimer 1990, de Lamirande, Leclerc et al. 1997). Utilizing these parameters, we applied a computerized system to measure, quantify, and categorize sperm; this system is known as Computer-Assisted

Sperm Analysis (CASA). We took advantage of this system to determine the effects of CC on sperm motility and hyperactivity.

The ultimate test of CC's efficacy on sperm's ability to fertilize was to perform an *in vitro* fertilization (IVF) experiment. Experiments were designed to allow observation of the effect of CC on sperm (a), on oocytes (b), and on sperm and oocytes together (c).

3.2 Results – Aim 2.1

3.2.1 Investigation of AMPK in Murine Sperm

To investigate the presence of AMPK in mouse sperm, an initial test by Western blotting was performed on pelleted CD1 mouse cells. Protein was extracted under varying conditions of detergent stringency: Triton, RIPA, and SDS. The increasing stringency of extraction was to ensure all parts of the sperm were broken down and the protein extracted. Approximately 2×10^6 cells were loaded per lane. Western analyses blot were run using a polyclonal AMPK antibody at a concentration of 1:1000. AMPK predicted molecular weight (MW) in somatic cells is ~62kDa. HEK293T cells (human embryonic kidney cells, a cell line known to contain AMPK) were included to validate the antibody, and a total cell extract from murine C2C12 cell (myoblasts) was also used to validate this antibody to recognize AMPK in mouse cells. According to this initial test, AMPK was not detected in mouse sperm (data not shown). In order to confirm these results, two distinct western blots were performed next; three individual mice were sacrificed and their sperm extracted under SDS conditions. Roughly 2×10^6 mouse sperm cells were loaded per lane in each gel; in parallel, 7.5×10^4 293T cells were loaded as positive control. One blot was probed using a monoclonal AMPK- α antibody (clone E6, Fig. 14a) also at a concentration of 1:1000 while the other was probed with the polyclonal

AMPK- α at the same concentration (blot not shown). As the Western blot in Figure 14(a) indicates, the monoclonal antibody recognized AMPK in both positive controls, human and mouse cells, at the expected MW of 62kDa. However, and despite a long exposure prior to film developing, AMPK was nearly undetectable in mouse sperm samples even when a monoclonal antibody is utilized.

In order to further assess these findings, AMPK immunoprecipitations were performed, followed by Western blotting using C2C12 lysate as a positive control, and 293T cells as a secondary biological control; in some cases, IP reactions for AMPK from 293T were carried out in parallel. Each IP sample had an accompanying input sample. For 293T cells, a RIPA-soluble extract was prepared and about 7.5×10^4 cells were loaded per lane; the IP sample contained $\sim 4 \times 10^6$ cells. To each lane, approximately 1.5×10^6 of mouse sperm cells were loaded as murine RIPA-extracted input samples while $\sim 2.5 \times 10^7$ mouse sperm cells were loaded as IP samples.. Although this experiment was run multiple times using both a monoclonal and a polyclonal antibody (both at 1:100), as well as with secondary antibodies against either immunoglobulin light chain (L) only or high and light (H+L) chains together, AMPK was not detectable in mouse sperm under the several experimental conditions tested. Figures 14 (c & d) are representative of what was found most often throughout multiple experiments. The Western blot performed using monoclonal antibody suggested that there may be barely-detectable amounts of AMPK in mouse sperm, but IP blots suggest that AMPK may not be in mouse sperm, at least not consistently in all mouse sperm samples analyzed. In very few cases, it could only be detected when sperm was highly concentrated and/or at high exposure times prior

to developing, implying that there may only be small amounts, if any, of AMPK present in mouse sperm.

3.3 Results – Aim 2.2

3.3.1 Effect of Compound C on Sperm Signaling Events

Regardless of whether or not AMPK was present in mouse sperm, it was necessary to discern if CC effected sperm signaling. Utilizing the pathway in Figure 3 as a model, it was essential to investigate the two primary areas of the pathway that could be affected: PKA-dependent phosphorylation and tyrosine phosphorylation (PY) events. It has been well established that phosphorylation of PKA substrates is indicative of the activation of this known pathway following upstream events (bicarbonate-dependent activation of soluble adenylate cyclase, cAMP, and then PKA) (Visconti, Moore et al. 1995, Muratori, Luconi et al. 2009, Visconti 2009). Downstream of PKA is tyrosine phosphorylation. PY signaling is indicative of whether or not sperm have achieved a capacitated state (Visconti, Bailey et al. 1995). The two signaling events act as a point of reference within the sperm signaling pathways, thus allowing for a rational evaluation of TSSK1 in the signaling events conducive to sperm capacitation and fertilizing ability. In this Aim, the effect of Compound C on these signaling cascades was analyzed.

Looking at signaling trends, rather than for a specific band, from the PKA substrate blot (Fig. 15a) it can be seen that there was no significant change in PKA signal strength even as the concentration of CC increased from 1 μ M to 100 μ M. The lower blot (Fig. 15b), PY, showed a different trend than the PKA blot; as concentrations of CC increased, PY signaling decreased, indicating a dose-dependent inhibitory response. While there are clear differences between the controls –Cap shows a darker signal

(indicative of successful capacitation) than non-cap (where almost no signal is detected), the DMSO-treated sample appears to produce similar signaling as the Cap, but starting around 10 μ M, a significant decrease in signaling can be observed. Based on these results, the signaling trend suggested that CC was acting on a target(s) probably downstream of PKA but upstream of PY. Figure 15c is the phosphorylated hexokinase. In murine sperm PY blots, the phosphorylated hexokinase may be used as an internal loading control as it is constitutively phosphorylated on tyrosine (Asquith, Baleato et al. 2004).

3.3.2 Computer-Assisted Sperm Analysis

Using the CASA system described in Figure 16, total motility and hyperactivity of sperm (average) was evaluated when sperm were exposed to CC at varying concentrations. Both Cap and Cap + DMSO (at 1%) were used controls, providing a baseline with which to compare the CC-treated cells. As per the parameters outlined by Goodson et al. (2011) based on the kinematics described by WHO (2010), sperm underwent capacitation and were subjected to CC treatments; 20 μ l of sample containing approximately $2-4 \times 10^5$ mouse sperm cells were then removed from the experimental tubes and introduced to the 2-chambered slides for data capturing and analysis. From the CASAnova analysis, the total number of motile sperm was calculated for each treatment. The average of sperm motility and hyperactivation acquired from several experiments (N>4) was calculated, and Figure 16 shows the resulting bar graph. The results were analyzed using ANOVA to calculate significant variance; post transformation, data underwent the Tukey test. The resulting motility of sperm exposed to CC at either 50 μ M or 100 μ M was significantly less than that of the controls (compared against the Cap control). From those motile cells, the average number of hyperactive cells was calculated

using the CASAnova software. The lower graph of Figure 16 displays these results; again the numbers were analyzed using ANOVA and post-test Turkey test. From the graph, it can be seen that there was a significant decrease in hyperactive sperm when exposed to 30 μ M or higher concentrations of Compound C.

One area that had yet to be explored was whether the effect of CC on sperm motility was reversible. According to numerous sources, the inhibitor should be reversible (Hurtado de Llera, Martin-Hidalgo et al. 2012, Liu, Chhipa et al. 2014); however, under the couple of experimental conditions we tried in the current study (data not shown), it was not feasible to reverse the effects of the inhibitor. Replicates of the following experiments were tried: following capacitation with exposure to CC, a 10% of the sample was removed and put into fresh Cap media without CC and then incubated for another hour. After the hour of incubation, 20 μ l was inspected using CASA. Two challenges arose due to this experimental design: there were not enough sperm cells to analyze, and none of the sperm was moving, even at low concentrations of the inhibitor, indicating that serial dilution was an ineffective method of reducing the concentration of CC in the media. The second method attempted included a centrifugation and wash using Cap media following incubation with CC and capacitation. This wash method was more successful than that of serial dilution, but even after allowing the sperm to incubate in the clean media for four hours beyond capacitation (with CASA analysis or video microscopy at 20x magnification every 30-60 minutes) there was no significant movement or indication of recovered motility. However, in the videos taken some sperm cells displayed a “shivering” motion. Due to this “shiver,” I wondered whether or not the

sperm undergo a type of paralysis, however there is no evidence in the sperm/CC literature that would support this thought.

3.3.3 Compound C and the Acrosome Reaction

Next, in Figure 17 we looked at the effects of CC on the AR. The controls included Cap samples, and Cap + DMSO (at 1%), both with and without ionophore; this ensured that the ionophore was functioning properly while acting as an experimental control for comparison, and to control for the ionophore effect. Using fluorescent FITC-PNA staining (as per the protocol in Fig. 6) and DIC/green channel ('GFP filter') microscopy, the number of acrosome-reacted versus unreacted cells was quantified as per Yoshida (Yoshida, Ito et al. 2010). Looking at the upper panel in Fig. 16, the control Cap cells (without ionophore) displays numerous intact acrosomes –the green slivers indicated by PNA staining under the GFP filter are the intact acrosomes of individual mouse sperm cells. The merged overlay and the region of interest (ROI) give clear images of fully intact acrosomes and initial stages of the AR. From this panel, it can also be seen that very few (approximately two cells in this image) have already undergone the AR (indicated by the lack of GFP in the acrosome). This was considered “spontaneous acrosome-reacted” sperm. Upon treatment with CC, there is a significant decrease in PNA staining; the merged overlay and ROI are a visual representation of acrosome-reacted spermatozoa. Although it was anticipated that CC could inhibit the AR as concentrations increased, the opposite effect occurred as is evident in Fig. 16 (bar graph). Instead of inhibiting the AR, CC seems to have had the same or a similar effect as would a calcium ionophore, suggesting that the inhibitor is actually acting upon a kinase involved in induction of mouse sperm AR. The data shown is of treatments in Cap

conditions but without an ionophore (except in the indicated treatment, bar on far right).

The bar graph also provides a few other important pieces of data: T=0 is the spontaneous AR; ~55% of sperm undergo spontaneous AR in average among several experiments performed (which is also consistent with published observation by other authors).

Interestingly, CC at concentrations $\geq 10\mu\text{M}$ display similar rates of AR in comparison to the Cap with ionophore treated sample. ANOVA statistical analysis was performed and a post-test Fisher analysis was performed to compare the data against the T=0 result. From this analysis, all treatments with CC and the ionophore are statistically significant compared to T=0. Viability was run in parallel (data not shown) using the Live/Dead® Sperm Viability kit to ensure CC was not affecting sperm viability.

3.3.4 *In Vitro* Fertilization

Finally, to test the effect of CC on the sperm's ability to fertilize, *in vitro* fertilization (IVF) was performed. Using a slightly different experimental design than in previous experiments, a range of treatments was performed to test a few different possible scenarios. Firstly, a 1x TYH capacitating medium containing BSA (4mg/ml) and HCO_3^- (15mM) was prepared. This capacitating media was prepared 13 hours prior to use and was kept in a CO_2 incubator at 37°C to allow appropriate CO_2 exchange. The day of the experiment, mTYH NC media was prepared. Experimental treatments were as follows: Capacitating media plus DMSO (at 1%), sperm pre-incubated in TYH Cap media with $30\mu\text{M}$ CC or $100\mu\text{M}$ of CC, oocytes pre-incubated with $100\mu\text{M}$ CC, sperm pre-incubated with H89 at $50\mu\text{M}$, and sperm not only pre-incubated with $100\mu\text{M}$ of CC, but also co-incubated – meaning that CC was added to the dish containing both the sperm and oocytes for a final concentration of $100\mu\text{M}$ CC maintained throughout the entire

experiment. Of equal importance to this experiment was that with sperm cells (or oocytes) considered “pre-incubated,” they underwent a wash using their respective medium prior to incubation with oocytes (or sperm) in order to minimize carry-over of CC persisting in the experimental environment. After initiation of the sperm-oocytes co-incubation, the dishes were kept in the CO₂ incubator at 37°C; four hours later, the oocyte/cumulus complex was washed and further incubated for an additional 16-20 hours. The next day, the oocytes were examined at 60X magnification and the number of 1-cell and 2-cell oocytes was counted. Figure 18 shows images representative of the majority of fertilization stages (or the lack thereof) seen following incubation. The DMSO-treated sperm (control) typically incurred high rates of fertilization, as depicted by embryos in the 2-cell stage (Fig. 18a). Oocytes incubated with sperm exposed to CC at 30μM showed 2-celled embryos (18b), but only 1-celled oocytes at 100μM (18c). It was interesting that in Figure 18(d) the concentration of cumulus cells surrounding the oocyte was very high. According to the literature, cumulus cells contain AMPK (Santiquet, Sasseville et al. 2014); CC inhibited the cumulus expansion normally experienced by cumulus cells prior to fertilization. Without cumulus expansion, the sperm are unable to reach the oocyte. Figure 18e demonstrates that even though the oocytes were exposed to CC, the washing step in the protocol decreases the concentration of CC enough to allow for cumulus expansion and occasional fertilization. The negative control, sperm pre-incubated with H89, a known PKA inhibitor, showed a low number of fertilized cells, as expected (18f).

As described, the number of 1-cell and 2-cell oocytes was quantified. Oocytes in the 1-cell stage were considered unfertilized and those in the 2-cell stage were

categorized as fertilized. In some instances oocytes were considered “degraded” and were put into the “unfertilized” category as they were unable to be fertilized due to oocyte deformation. The averages of three experiments are expressed in the accompanying graph in Figure 18. The graph indicates successful fertilization of oocytes with DMSO-treated sperm cells (positive control), and a significantly low amount of fertilized oocytes in sperm cells incubated in H89 (negative control). At the lower concentration of CC, some oocytes were fertilized; at higher concentrations and when co-incubated with CC, almost no oocytes were fertilized. When oocytes were pre-incubated with CC, there was some fertilization, significantly higher than when the sperm was pre-incubated, but less than the control and when sperm was pre-incubated at 30 μ M. It is likely that when the oocytes were washed following pre-incubation, the concentration of CC remaining with the cumulus cells and oocytes was diminished to a level that would allow sperm to fertilize the oocytes. To determine statistical significance of fertilization rates, the data was tested using ANOVA, followed by the Tukey’s test.

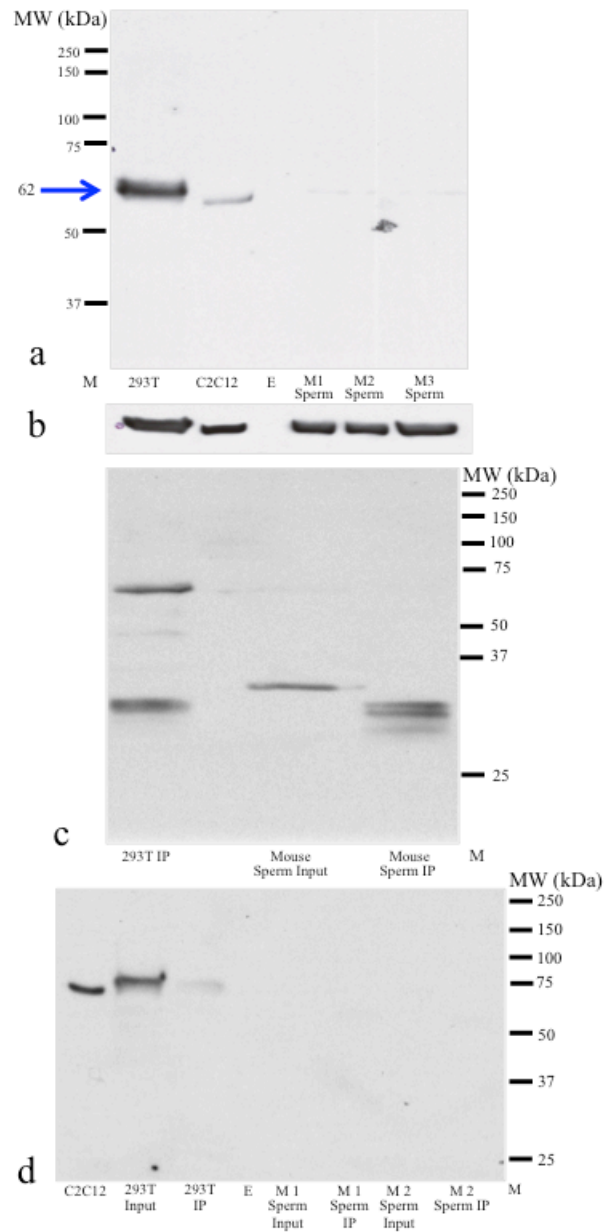


Figure 14: Investigation of AMPK in Murine Sperm. Analysis of the presence of AMPK in mouse sperm by Western blotting and immunoprecipitation. Mouse sperm samples extracted under SDS conditions and probed against a monoclonal AMPK antibody; a C2C12 mouse myoblasts lysate was used as a positive control in addition to the 293T RIPA-soluble extracted cells as a biological control (a) N=3. Blot (b) is of the corresponding tubulin blot (loading control) N=3. Mouse sperm extracts analyzed by immunoprecipitation using anti-AMPK (polyclonal) (c & d). N =1, N=2, respectively. E: empty lane.

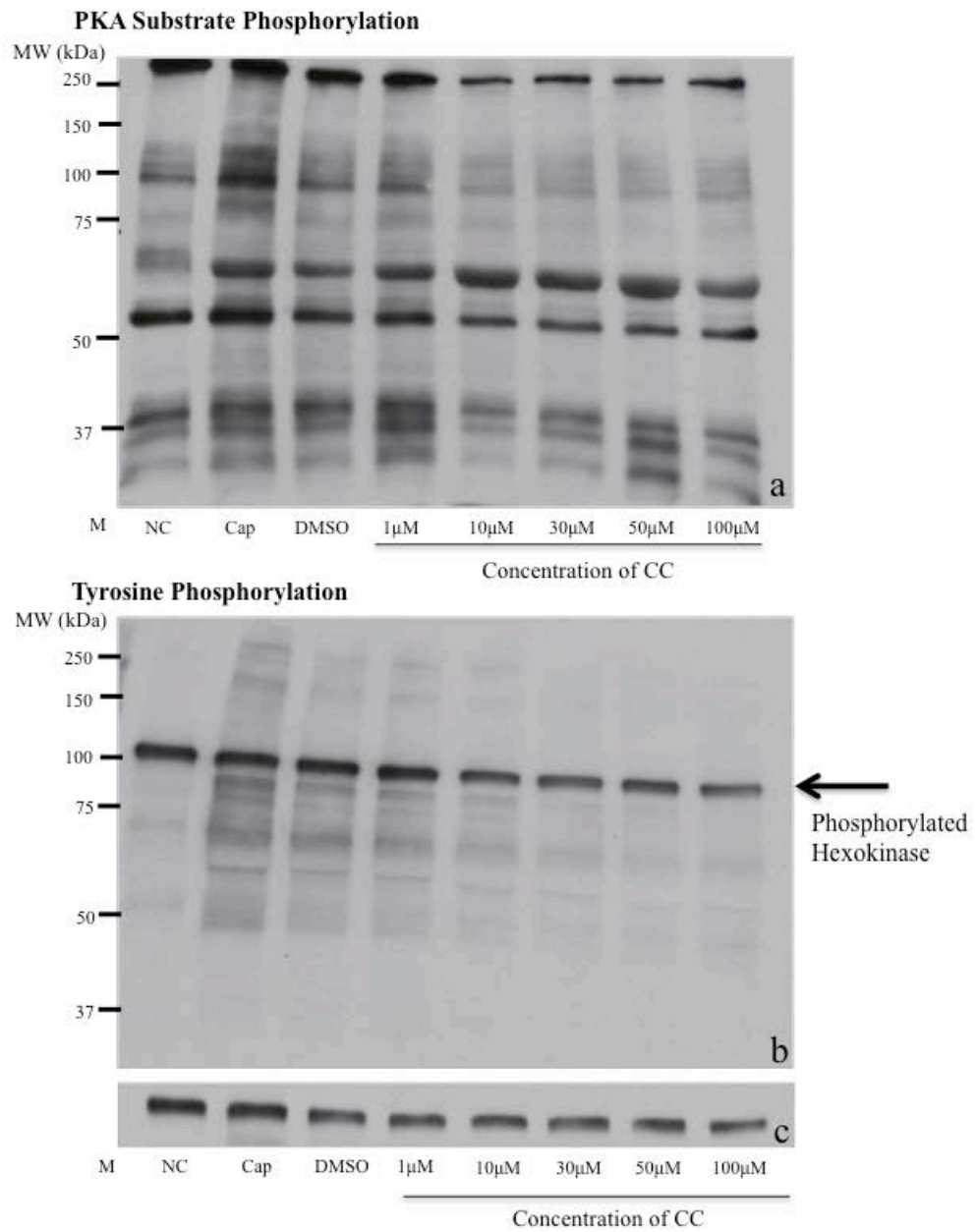


Figure 15: Effect of Compound C on Sperm Signaling Events. Analysis of sperm signaling events in response to CC. a) Western blot showing phosphorylated PKA substrates. Tyrosine phosphorylation (PY) signaling is depicted in blot (b). Blot (c) is phosphorylated hexokinase; it acts as an internal loading control since it is constitutively phosphorylated on tyrosine in mouse sperm. Images shown are representative of experiments run four times or more.

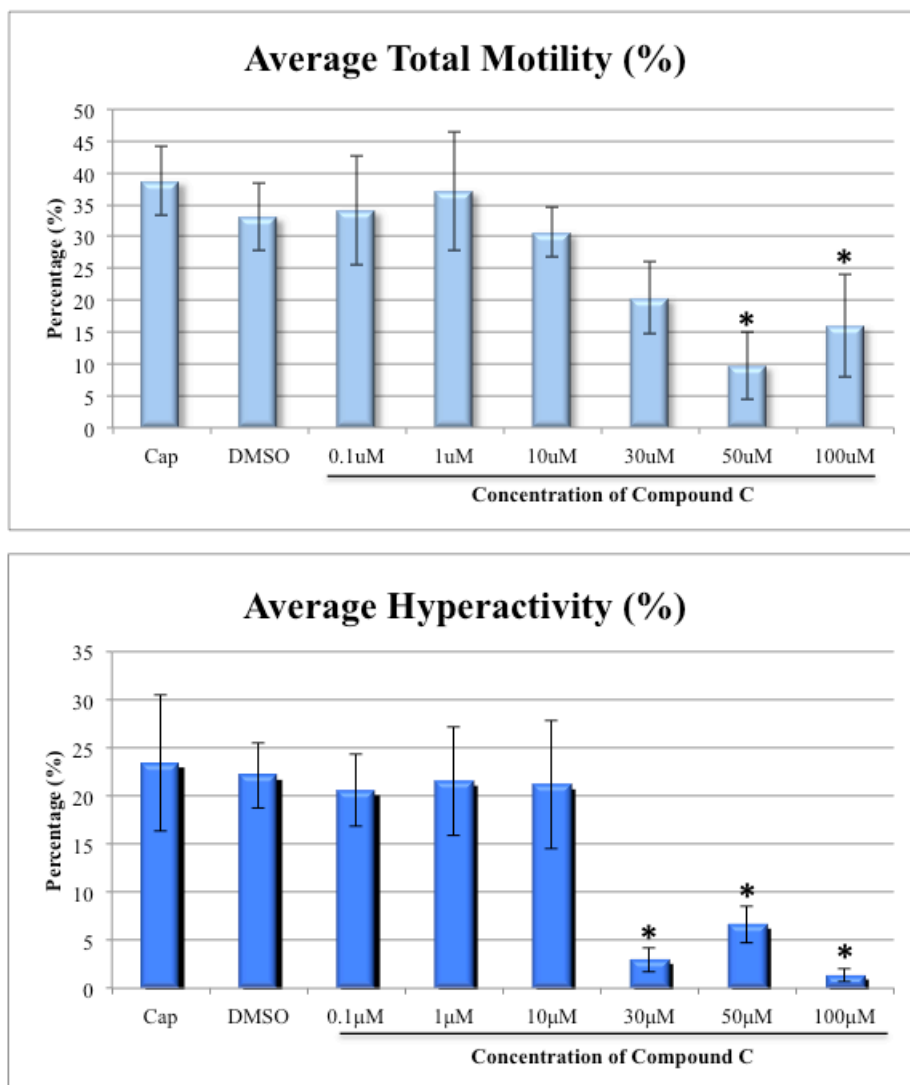


Figure 16: Computer-Assisted Sperm Analysis on Motility and Hyperactivation of Sperm in the Presence of Compound C. CASA analysis of the dose-dependent effect of CC on mouse sperm motility. The top graph shows the average total motility of mouse sperm (expressed as a percentage of Cap controls without CC) following exposure to varying concentrations of CC. The lower graph shows a percentage that represents the number of hyperactive sperm out of the percentage of motile sperm. (*) indicates significant differences found at $p < 0.05$ compared to controls. N=3

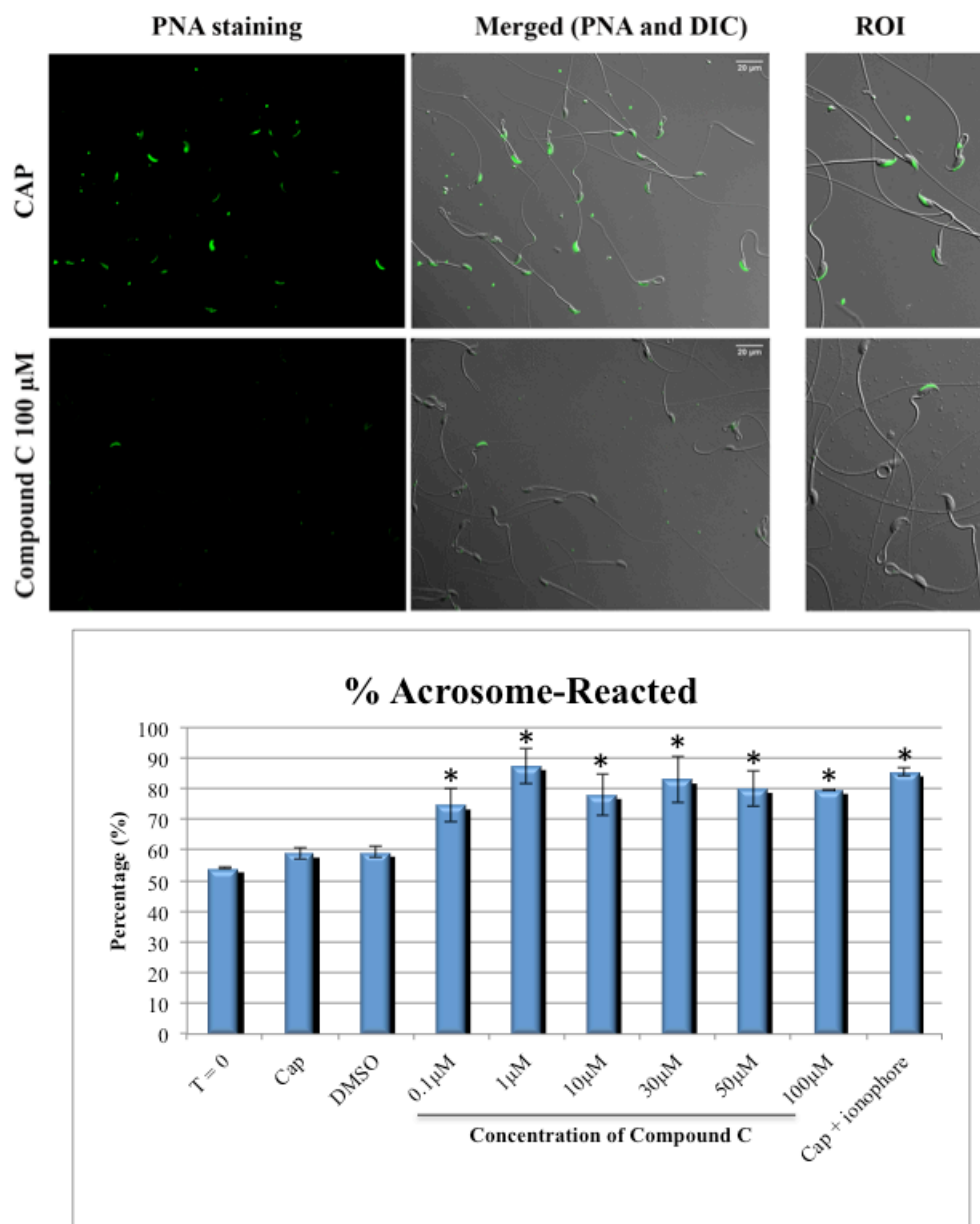
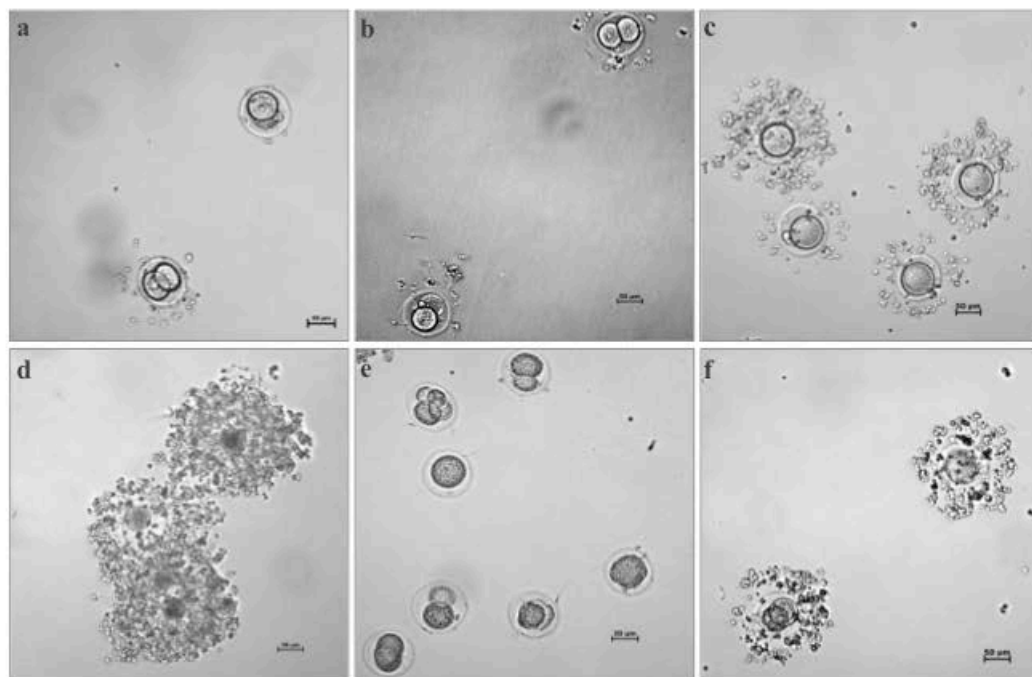


Figure 17: The Effect of Compound C on the Acrosome Reaction. Effect of CC on the occurrence of spontaneous acrosome reaction (AR) in mouse sperm analyzed by PNA-staining. Representative fluorescence images depicting spontaneous AR upon treatment with CC (upper panel), were quantified by cell counting as percentage of the sperm AR found under capacitating conditions at time 0 (Cap, T=0) as a control (lower panel). Significant differences at $p < 0.05$ compared to control are indicated as (*). N= 2-3, A=600x



Percent Fertilized (%)

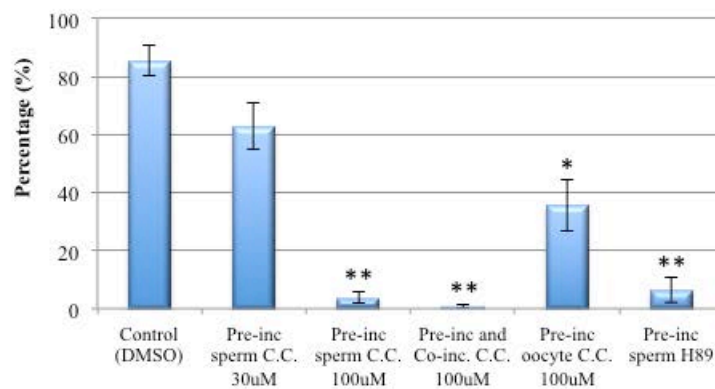


Figure 18: *In Vitro* Fertilization. Effect of CC on fertilization rates *in vitro*. Sperm was pre-incubated with: (a) 1% DMSO, (b) CC at 30 μ M, (c) CC at 100 μ M, or (f) with H89 at 50 μ M for 15 minutes prior to capacitation. Separate IVF reactions were set up in which sperm was pre-incubated with CC, and CC also added to the oocyte/sperm mixture (d) to maintain a constant concentration of 100 μ M. Additionally, mouse oocytes were pre-incubated with 100 μ M CC prior to exposure to the sperm (e). Representative images for each treatment are shown (N=2-3). The bar graph shows the rate of fertilization (% IVF) following the treatments listed above. * $p < 0.05$, ** $p < 0.01$ when compared to the DMSO control.

CHAPTER 4

CONCLUSIONS, DISCUSSION, & FUTURE DIRECTIONS

4.1 Conclusions & Discussion

The major finding of the *in vitro* studies performed for this Thesis is that the ADP-Glo™ assay is indeed suitable for use with our kinase of interest, TSSK1, even at low concentrations, making the feasibility of HTS and the possibility of screening a few thousand inhibitors against TSSK1 a certainty. As mentioned previously, the details outlined by Brooks, among others, indicate the numerous steps that must be taken prior to HTS to optimize the chances of finding the desired outcome – which in this case is a TSSK1 kinase inhibitor suitable for drug development (Brooks 2012). Based on the assays performed, nearly all required steps were met: all reagents were acquired, instrumentation was arranged, the assay concept was corroborated (see initial figures for this chapter), and the assay itself was optimized and validated.

One of the first steps of narrowing inhibitor choices was undertaken during this project. By testing the activity of TSSK1 against a variety of inhibitors in our mid-throughput screening assay, results indicated significant inhibition in comparison to the control. As discussed, one such inhibitor showing promise was Compound C (CC). Although SKI-606 showed a comparable inhibitory effect, since it was a broad kinase inhibitor of Src and tyrosines, it was agreed that a more specific inhibitor should be investigated. Besides the evidence the assay provided, the several sources of literature indicated that CC was a potent, selective, and potentially reversible inhibitor of AMPK (Zhou, Myers et al. 2001, Hurtado de Llera, Martin-Hidalgo et al. 2012, Liu, Chhipa et al. 2014, Nguyen, Alves et al. 2014).

One clear observation was that both CC (a known serine/threonine kinase inhibitor) and SKI-606 (a known tyrosine kinase inhibitor) could be equally inhibiting TSSK1 activity. This fact, which had been also considered among the expected outcomes of testing such small battery of inhibitors, indicated that it is highly unlikely that CC was specific enough for further use in drug design against TSSK1 activity. However, the study was continued using this kinase inhibitor as a good indicator of potent TSSK1 inhibition *in vitro*, and paved the way for *in vivo* studies. Also, because these assays were conducted prior to performing *in vivo* work, and because there was no strong evidence in the literature on the absence or presence of AMPK in mouse sperm, there was no way to know if CC was inhibiting AMPK or TSSK1. However, data presented here (best depicted in Figure 13) clearly supports the notion that CC can inhibit TSSK1 *in vitro*, even at very low concentrations.

Due to its efficacy *in vitro*, *in vivo* assays were pursued, and the CC effect studied in mature mouse spermatozoa. However, caution was taken when considering the molecule as an *in vivo* inhibitor as per warnings found in Liu et al., which stated their lack of confidence in CC being a selective inhibitor of AMPK. But, because Liu did not perform *in vitro* studies on their cell line, it was decided to be a strong rationale for pursuing our analyses *in vivo*.

The chief findings of these studies included (a) a very low abundance (if present) of AMPK in murine sperm; (b) the initial placement of a Compound C-sensitive kinase, which we hypothesize to be TSSK1, in the sperm biochemical pathway conducive to capacitation; (c) the effects of CC on sperm motility, viability, and acrosome reaction; and (d) the effect of CC in the fertilization process. Extensive literature searches were

performed to look into the presence or absence of AMPK in mouse sperm, although there is scarce published data on the subject, at least for the mouse model. As discussed in the Results section, we were able to detect a faint signal that was barely detectable following a long exposure in a Western blot probed with a monoclonal antibody. To increase the chances of detecting mouse sperm AMPK –by concentrating the sperm sample –an IP was performed, and despite multiple attempts with both poly- and monoclonal antibodies against alpha-AMPK, the catalytic subunit of AMPK, and use of both IgA and IgG beads, no murine signal was produced on the corresponding Western blot. These findings indicate that while there may be AMPK present in mouse sperm, it is at an extremely low concentration, which is in agreement with recent data by another lab using a different set of antibodies (Tartarin, Guibert et al. 2012). With this new insight, and under the presupposition that there are likely almost undetectable levels of AMPK in mouse sperm, the small-molecule inhibitor, CC was investigated in known sperm signaling pathways.

As displayed in Figure 3, there are well-defined biochemical pathways in mature spermatozoa that are activated during capacitation (Visconti 2009). In our studies, as shown in Figure 15, a concentration curve of CC did not show any obvious change in the levels of phosphorylated PKA substrates, typically used as an indicator of PKA activation. In contrast, PY signaling was impacted. These findings would suggest that TSSK1 may be signaling upstream of PY, and thus PY-dependent phosphorylation (but not PKA), would be inhibited by Compound C. Alternatively, although not well understood at this time, a role for TSSK1 autophosphorylation in this cascade of events cannot be ruled out. According to early studies by Kueng et al. (1997) and others, and also based on *in vitro* data presented in this Thesis (Figure 9), TSSK1 is able to undergo

autophosphorylation (Kueng, Nikolova et al. 1997, Jaleel, McBride et al. 2005, Spiridonov, Wong et al. 2005). In this regard, TSSK1 autophosphorylating activity could be altered by CC, thus affecting signaling events related to sperm capacitation.

The next conclusion was on the effect of Compound C on the occurrence of sperm spontaneous AR. As discussed in the Introduction section of this Thesis, the current model proposes that the acrosome reaction occurs when the sperm fuses with the oocyte and the enzyme-containing pocket is released. In this study the effect of CC increased the rate of AR. This indicated that instead of inhibiting the AR, some kinase(s) was being activated upon treatment with CC. It is interesting to note that our results, looking into the occurrence of *spontaneous* AR, contradict observations by other authors in boar sperm: according to the only source we found that investigated the effects of CC on mammalian sperm AR, the *ionophore-induced* AR is not affected by CC (Hurtado de Llera, Martin-Hidalgo et al. 2013). However, another source examined the effects of CC on oocytes in mice, and found that the AMPK inhibitor produced an opposite effect, stating that CC “increased the frequency of oocyte activation” (Downs, Ya et al. 2010, Acton 2012). The findings by Downs, et al. are very informative because although it was found in a different cell type, they too experienced an opposite effect than predicted. Our data also shows that the effect of CC on AR can reach levels as high as those found when AR is induced by a calcium ionophore (Figure 17).

Performing a concentration curve with CC and subjecting the sperm to CASA analysis showed a severe decline in sperm motility. Consistent with recent studies by three independent research groups –Hurtado de Llera, Nguyen and Tartarin –who have investigated the effects of CC on boar, chicken, and mouse spermatozoa, respectively –

CC has shown inhibitory effects on sperm motility and hyperactivation. Since the present project also examined viability in parallel to CASA experiments, it was also confirmed by the aforementioned sources that viability decreased only when exposed to the inhibitor for either an extended period of time, or to high concentrations (100uM or higher). It is important to note that an area requiring additional research is the reversibility of CC in relation to sperm motility. As discussed in the Results section, we performed a series of tests to address this question; however we were unable to detect clear signs of recovery on motility in sperm that had been exposed to CC.

The critical final step of evaluating the effect of CC on spermatozoa was to test its effect on fertilization. Compound C induced significantly decreased rates of fertilization in mice, similar to the decreased rates of fertilization of Tartarin *et al.* (Tartarin, Guibert et al. 2012). This is the true test of inhibitor potency since it is considered the most relevant functional endpoint to the process of contraceptive development. Thus far, no other studies have been performed using CC on IVF. It was interesting and somewhat unexpected that IVF using sperm pre-incubated with 30uM CC was able to fertilize as much as it did; the CASA data indicated that although there was only ~20% sperm motility, there was less than 5% of hyperactivity. As discussed previously, sperm must achieve hyperactivity prior to fertilization. With such low percentages of motile, hyperactive sperm, it seemed unlikely that many oocytes could have been fertilized. However our data shows that the opposite may be true. Ideally, additional experiments, such as using a wider range of CC concentrations, could be performed. IVF tends to be a costly experiment in terms of time and lab resources, including male and female mice.

4.2. Future Directions

The remaining steps necessary for commencing HTS using TSSK1 on a larger set of kinase inhibitors library is beyond the scope of this project. Following the experiments performed, in the case of our lab, collaborations with the HTS Facility at UMass Worcester have been recently established to pursue such goal. Leaving aside the financial aspect that accompanies any project needing to utilize a specialty Facility, inhibitors must be selected based on the biochemical properties of the intended target. Although there have been preliminary steps taken toward creation of a biased library of a few thousands of inhibitors, the protocols are still underway. Additionally, a few biochemical parameters remain to be determined or calculated for TSSK1, such as the IC_{50} of the kinase (one experiment has been performed to generate an approximate calculation, but more replicates are necessary); establishment of a crystal structure of TSSK1 would be ideal to have. Our group is currently working on 3-D models of TSSK1, which should help on a more rationale design of a TSSK1-focused screening of kinase inhibitors by HTS.

In terms of our *in vivo* findings, many of the next steps needed for this project lie within the ability to detect the presence of AMPK in mouse sperm. Additional experiments should be devised towards detecting the regulatory subunits of AMPK in sperm, for instance, using antibodies against AMPK β and/or AMPK γ . Since AMPK α is the catalytic subunit, it seemed reasonable to test that specific subunit first. However, since it produced results that may be taken as contradictory, to some degree, when compared to published data on sperm from other species, testing the other two component

subunits of the AMPK complex would make for a more complete and thorough investigation of the presence or absence of AMPK in murine sperm.

To definitively test whether or not PKA is affected by CC, a radioactive kinase assay should be performed. Using P^{32} ATP, the activity of PKA's ability to phosphorylate the substrate Kemptide under conditions including CC could be quantitatively measured.

Further experiments in the realm of the acrosome reaction should be performed. As mentioned, the results that CC activated the AR were unexpected; if new experiments were performed and the results comparable, then it could be confirmed that CC indeed increases the rate of AR. However, further investigation into the biochemical reasoning for this occurrence would have to take place as well. Currently there is no evidence as to why or how this could happen so the biochemical aspect would have to be taken into consideration. Although it would take a great deal of time to optimize the conditions, fluorescence activated cell sorting (FACS) is another option for analysis. Once the parameters were established, FACS would be a way to quickly determine whether or not a cell had released its acrosome contents and the data could be plotted for analysis.

Additional experiments to investigate whether or not sperm can regain motility and/or achieve a hyperactive state following exposure to CC would be another area where more research is needed. Several sources indicate that CC should be a reversible inhibitor, however our attempts in this area have indicated otherwise. An interesting experiment to look into this issue would be to perform an IVF experiment and then wash the oocyte/cumulus cells/sperm cell complex following the 4 hour incubation and see if replacing the media would allow sperm to regain its fertilization ability.

In summary, we have been able to further validate the use of a homogeneous kinase assay to assess TSSK1 activity *in vitro*, and have found experimental conditions for screening of large libraries of TSSK1 inhibitors by HTS. Furthermore, we have applied several functional *in vivo* assays for validating such inhibitor(s) as potential blocker(s) of TSSK1 function. In addition, we provide here valuable information on AMPK being present at very low levels in mouse sperm when compared to data provided by other authors who have analyzed the role of CC on the AMPK pathway in other species. Finally, we have documented very important observations on the effect of Compound C (also known as dorsomorphin or BML-275) on sperm signaling pathways conducive to its fertilizing ability. Overall, this Thesis provides strong support on TSSK1 as a potential target for development of male contraceptives, as well as several rationale endpoints for validation of TSSK1 potential inhibitors using both *in vitro* and *in vivo* settings.

BIBLIOGRAPHY

- Acton, A. Q. (2012). Germ Cells: Advances in Research and Application. Atlanta, GA, ScholarlyEditions.
- Aitken, R. J., M. Paterson, H. Fisher, D. W. Buckingham and M. van Duin (1995). "Redox regulation of tyrosine phosphorylation in human spermatozoa and its role in the control of human sperm function." J Cell Sci **108** (Pt 5): 2017-2025.
- Asquith, K. L., R. M. Baleato, E. A. McLaughlin, B. Nixon and R. J. Aitken (2004). "Tyrosine phosphorylation activates surface chaperones facilitating sperm-zona recognition." J Cell Sci **117**(Pt 16): 3645-3657.
- Barros, C., J. M. Bedford, L. E. Franklin and C. R. Austin (1967). "Membrane vesiculation as a feature of the mammalian acrosome reaction." J Cell Biol **34**(3): C1-5.
- Bielke, W., R. J. Blaschke, G. C. Miescher, G. Zurcher, A. C. Andres and A. Ziemiecki (1994). "Characterization of a novel murine testis-specific serine/threonine kinase." Gene **139**(2): 235-239.
- Breitbart, H. and Z. Naor (1999). "Protein kinases in mammalian sperm capacitation and the acrosome reaction." Rev Reprod **4**(3): 151-159.
- Brooks, H. B., Geeganage, S., Kahl, S.D., Montrose, C., Sittampalam, S., Smith, M.C., Weidner, J. R. (2012). Assay Guidance Manual.
- Bucko-Justyna, M., L. Lipinski, B. M. Burgering and L. Trzeciak (2005). "Characterization of testis-specific serine-threonine kinase 3 and its activation by phosphoinositide-dependent kinase-1-dependent signalling." FEBS J **272**(24): 6310-6323.
- Chang, M. C. (1951). "Fertilizing capacity of spermatozoa deposited into the fallopian tubes." Nature **168**(4277): 697-698.
- Control, C. f. D. (2015, Feb. 24, 2015). "Contraception." 2015.
- Dan, J. C. (1952). "Studies on the Acrosome. I. Reaction to Egg-Water and Other Stimuli." Biological Bulletin **103**(1): 54-66.
- de Lamirande, E., P. Leclerc and C. Gagnon (1997). "Capacitation as a regulatory event that primes spermatozoa for the acrosome reaction and fertilization." Mol Hum Reprod **3**(3): 175-194.
- Dorman, E. and D. Bishai (2012). "Demand for male contraception." Expert Rev Pharmacoecon Outcomes Res **12**(5): 605-613.

- Downs, S. M., R. Ya and C. C. Davis (2010). "Role of AMPK throughout meiotic maturation in the mouse oocyte: evidence for promotion of polar body formation and suppression of premature activation." Mol Reprod Dev **77**(10): 888-899.
- Garner, D. L. and L. A. Johnson (1995). "Viability assessment of mammalian sperm using SYBR-14 and propidium iodide." Biol Reprod **53**(2): 276-284.
- Garside, D., Gebril, A., Alsaadi, M., Nimmo, N., Mullen, A., Ferro, V. (2013). "An Update on the Potential for Male Contraception: Emerging Options." Journal of Contraception **2013**:4: 1-11.
- Hao, Z., K. N. Jha, Y. H. Kim, S. Vemuganti, V. A. Westbrook, O. Chertihin, K. Markgraf, C. J. Flickinger, M. Coppola, J. C. Herr and P. E. Visconti (2004). "Expression analysis of the human testis-specific serine/threonine kinase (TSSK) homologues. A TSSK member is present in the equatorial segment of human sperm." Mol Hum Reprod **10**(6): 433-444.
- Hardie, D. G. (2015). "AMPK: positive and negative regulation, and its role in whole-body energy homeostasis." Curr Opin Cell Biol **33**: 1-7.
- Hasuwa, H., Y. Muro, M. Ikawa, N. Kato, Y. Tsujimoto and M. Okabe (2010). "Transgenic mouse sperm that have green acrosome and red mitochondria allow visualization of sperm and their acrosome reaction in vivo." Exp Anim **59**(1): 105-107.
- Hurtado de Llera, A., D. Martin-Hidalgo, M. C. Gil, L. J. Garcia-Marin and M. J. Bragado (2012). "AMP-activated kinase AMPK is expressed in boar spermatozoa and regulates motility." PLoS One **7**(6): e38840.
- Hurtado de Llera, A., D. Martin-Hidalgo, J. E. Rodriguez-Gil, M. C. Gil, L. J. Garcia-Marin and M. J. Bragado (2013). "AMP-activated kinase, AMPK, is involved in the maintenance of plasma membrane organization in boar spermatozoa." Biochim Biophys Acta **1828**(9): 2143-2151.
- Ikawa, M., S. Yamada, T. Nakanishi and M. Okabe (1999). "Green fluorescent protein (GFP) as a vital marker in mammals." Curr Top Dev Biol **44**: 1-20.
- Jaleel, M., A. McBride, J. M. Lizcano, M. Deak, R. Toth, N. A. Morrice and D. R. Alessi (2005). "Identification of the sucrose non-fermenting related kinase SNRK, as a novel LKB1 substrate." FEBS Lett **579**(6): 1417-1423.
- Kogan, P. and M. Wald (2014). "Male contraception: history and development." Urol Clin North Am **41**(1): 145-161.
- Kueng, P., Z. Nikolova, V. Djonov, A. Hemphill, V. Rohrbach, D. Boehlen, G. Zuercher, A. C. Andres and A. Ziemiecki (1997). "A novel family of serine/threonine kinases participating in spermiogenesis." J Cell Biol **139**(7): 1851-1859.

Laemmli, U. K. (1970). "Cleavage of structural proteins during the assembly of the head of bacteriophage T4." Nature **227**(5259): 680-685.

Leclerc, P., E. de Lamirande and C. Gagnon (1997). "Regulation of protein-tyrosine phosphorylation and human sperm capacitation by reactive oxygen derivatives." Free Radic Biol Med **22**(4): 643-656.

Li, Y., J. Sosnik, L. Brassard, M. Reese, N. A. Spiridonov, T. C. Bates, G. R. Johnson, J. Anguita, P. E. Visconti and A. M. Salicioni (2011). "Expression and localization of five members of the testis-specific serine kinase (Tssk) family in mouse and human sperm and testis." Mol Hum Reprod **17**(1): 42-56.

Liu, X., R. R. Chhipa, I. Nakano and B. Dasgupta (2014). "The AMPK inhibitor compound C is a potent AMPK-independent antiglioma agent." Mol Cancer Ther **13**(3): 596-605.

Lohiya, N. K., I. Alam, M. Hussain, S. R. Khan and A. S. Ansari (2014). "RISUG: an intravasal injectable male contraceptive." Indian J Med Res **140** **Suppl**: S63-72.

Manning, G., D. B. Whyte, R. Martinez, T. Hunter and S. Sudarsanam (2002). "The protein kinase complement of the human genome." Science **298**(5600): 1912-1934.

Mortimer, S. T. and D. Mortimer (1990). "Kinematics of human spermatozoa incubated under capacitating conditions." J Androl **11**(3): 195-203.

Mosher, W. D., J. Jones and J. C. Abma (2012). "Intended and unintended births in the United States: 1982-2010." Natl Health Stat Report(55): 1-28.

Muratori, M., M. Luconi, S. Marchiani, G. Forti and E. Baldi (2009). "Molecular markers of human sperm functions." Int J Androl **32**(1): 25-45.

Murdoch, F. E. and E. Goldberg (2014). "Male contraception: another Holy Grail." Bioorg Med Chem Lett **24**(2): 419-424.

Nakanishi, T., M. Ikawa, S. Yamada, M. Parvinen, T. Baba, Y. Nishimune and M. Okabe (1999). "Real-time observation of acrosomal dispersal from mouse sperm using GFP as a marker protein." FEBS Lett **449**(2-3): 277-283.

Nguyen, T. M., S. Alves, I. Grasseau, S. Metayer-Coustard, C. Praud, P. Froment and E. Blesbois (2014). "Central role of 5'-AMP-activated protein kinase in chicken sperm functions." Biol Reprod **91**(5): 121.

Nieschlag, E. (2010). "Clinical trials in male hormonal contraception." Contraception **82**(5): 457-470.

Okabe, M., M. Ikawa, K. Kominami, T. Nakanishi and Y. Nishimune (1997). "'Green mice' as a source of ubiquitous green cells." FEBS Lett **407**(3): 313-319.

Salicioni, A. M., Romano, F., and Visconti, P.E. (2012). "Testis-Specific Kinases in Male Fertility and as Targets for Contraception." American Pharmaceutical Review **July/Aug. 2012**.

Santiquet, N., M. Sasseville, M. Laforest, C. Guillemette, R. B. Gilchrist and F. J. Richard (2014). "Activation of 5' adenosine monophosphate-activated protein kinase blocks cumulus cell expansion through inhibition of protein synthesis during in vitro maturation in Swine." Biol Reprod **91**(2): 51.

Shang, P., W. M. Baarends, J. Hoogerbrugge, M. P. Ooms, W. A. van Cappellen, A. A. de Jong, G. R. Dohle, H. van Eenennaam, J. A. Gossen and J. A. Grootegoed (2010). "Functional transformation of the chromatoid body in mouse spermatids requires testis-specific serine/threonine kinases." J Cell Sci **123**(Pt 3): 331-339.

Spiridonov, N. A., L. Wong, P. M. Zerfas, M. F. Starost, S. D. Pack, C. P. Paweletz and G. R. Johnson (2005). "Identification and characterization of SSTK, a serine/threonine protein kinase essential for male fertility." Mol Cell Biol **25**(10): 4250-4261.

Suarez, S. S., D. F. Katz and J. W. Overstreet (1983). "Movement characteristics and acrosomal status of rabbit spermatozoa recovered at the site and time of fertilization." Biol Reprod **29**(5): 1277-1287.

Tartarin, P., E. Guibert, A. Toure, C. Ouiste, J. Leclerc, N. Sanz, S. Briere, J. L. Dacheux, B. Delaleu, J. R. McNeilly, A. S. McNeilly, J. P. Brillard, J. Dupont, M. Foretz, B. Viollet and P. Froment (2012). "Inactivation of AMPK α 1 induces asthenozoospermia and alters spermatozoa morphology." Endocrinology **153**(7): 3468-3481.

Tateno, H., D. Krapf, T. Hino, C. Sanchez-Cardenas, A. Darszon, R. Yanagimachi and P. E. Visconti (2013). "Ca²⁺ ionophore A23187 can make mouse spermatozoa capable of fertilizing in vitro without activation of cAMP-dependent phosphorylation pathways." Proc Natl Acad Sci U S A **110**(46): 18543-18548.

Ventela, S., M. Mulari, M. Okabe, H. Tanaka, Y. Nishimune, J. Toppari and M. Parvinen (2000). "Regulation of acrosome formation in mice expressing green fluorescent protein as a marker." Tissue Cell **32**(6): 501-507.

Visconti, P. E. (2009). "Understanding the molecular basis of sperm capacitation through kinase design." Proc Natl Acad Sci U S A **106**(3): 667-668.

Visconti, P. E., J. L. Bailey, G. D. Moore, D. Pan, P. Olds-Clarke and G. S. Kopf (1995). "Capacitation of mouse spermatozoa. I. Correlation between the capacitation state and protein tyrosine phosphorylation." Development **121**(4): 1129-1137.

Visconti, P. E., L. R. Johnson, M. Oyaski, M. Fornes, S. B. Moss, G. L. Gerton and G. S. Kopf (1997). "Regulation, localization, and anchoring of protein kinase A subunits during mouse sperm capacitation." Dev Biol **192**(2): 351-363.

- Visconti, P. E., G. D. Moore, J. L. Bailey, P. Leclerc, S. A. Connors, D. Pan, P. Olds-Clarke and G. S. Kopf (1995). "Capacitation of mouse spermatozoa. II. Protein tyrosine phosphorylation and capacitation are regulated by a cAMP-dependent pathway." Development **121**(4): 1139-1150.
- Wertheimer, E., D. Krapf, J. L. de la Vega-Beltran, C. Sanchez-Cardenas, F. Navarrete, D. Haddad, J. Escoffier, A. M. Salicioni, L. R. Levin, J. Buck, J. Mager, A. Darszon and P. E. Visconti (2013). "Compartmentalization of distinct cAMP signaling pathways in mammalian sperm." J Biol Chem **288**(49): 35307-35320.
- Xu, B., Z. Hao, K. N. Jha, Z. Zhang, C. Urekar, L. Digilio, S. Pulido, J. F. Strauss, 3rd, C. J. Flickinger and J. C. Herr (2008). "Targeted deletion of Tssk1 and 2 causes male infertility due to haploinsufficiency." Dev Biol **319**(2): 211-222.
- Yanagimachi, R. (1981). Fertilization and Embryo Development In Vitro: Mechanisms of Fertilization in Mammals. New York, Springer US.
- Yanagimachi, R. (1981). Mechanisms of Fertilization in Mammals. Fertilization and Embryonic Development In Vitro. New York, Springer US: 81-182.
- Yoshida, K., C. Ito, K. Yamatoya, M. Maekawa, Y. Toyama, F. Suzuki-Toyota and K. Toshimori (2010). "A model of the acrosome reaction progression via the acrosomal membrane-anchored protein equatorin." Reproduction **139**(3): 533-544.
- Zhou, G., R. Myers, Y. Li, Y. Chen, X. Shen, J. Fenyk-Melody, M. Wu, J. Ventre, T. Doebber, N. Fujii, N. Musi, M. F. Hirshman, L. J. Goodyear and D. E. Moller (2001). "Role of AMP-activated protein kinase in mechanism of metformin action." J Clin Invest **108**(8): 1167-1174.